STABILIZATION AND SOME OTHER PROPERTIES OF IMMOBILIZED GLYCOENZYMES

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Certificate

I certify that the work presented in this thesis has been carried out by **Mr. Shahid Husain** *under my Supervision and is suitable for submission for the award of* ***Ph.D. degree*** *in Biochemistry.*

M. Saleemuddin 25/11/94

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In the memory of my
Loving mother

To.....,

My Parents

(for leading me to intellectual horizons)

Farahdiba

(for her unending support)

Mahvash

(for making it worthwhile)

Acknowledgment

It is time to actually put into words the infinite gratitude that I owe to my supervisor Prof. M. Saleemuddin. He has been an epitome of patience and tolerance, a pedestal of strength and support, a fountainhead of of knowledge, forbearance and forgiveness. His knowledge of the subject, keen interest and patience will forever inspire me. For igniting the flame of knowledge and keeping it burning I will be forever thankful.

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To my wife, Farahdiba Husain, from the oceanic depths of my heart I thank her for being by my side while experiencing the rough and the smooth with perfect zeal, comfort and ease. For putting up an eversmiling front, uncomplaining and compromising attitude I would be indebted all her more.

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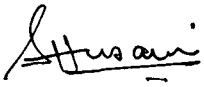

SHAHID HUSAIN

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Chapter I

INTRODUCTION

GENERAL

Living organisms have a remarkable ability to metabolize a wide range of compounds via multistep reactions catalyzed by enzymes Enzymes are proteins with remarkable capacity to accelerate rate of various reactions with high degree of specificity and high turnover under extremely mild conditions. The unique properties of enzymes have lead to their employment in food industry (Reed, 1975), clinical and analytical chemistry (Guilbault, 1970), preparative organic chemistry and medicine (Wolf and Ransberger, 1977; Bergsma, 1973)

The principal limitations in the large scale application of enzymes are their high cost, high susceptibility to inactivation, poor recoverability and reusability. Enzymes occur in nature as very heterogeneous mixtures of proteins and other constituents Isolation of enzymes in relatively pure form, involve tedious and expensive fractionation steps, while reusability of enzymes is seriously restricted by their water soluble nature. In order to circumvent the problems and to develop reusable catalysts suited for analytical, biomedical and industrial applications, immobilization of enzymes on insoluble polymeric supports was envisaged.

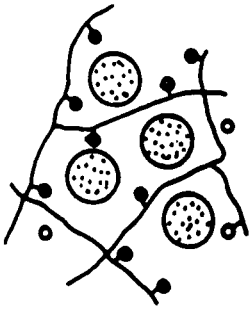
ENZYME IMMOBILIZATION

The term 'immobilized enzyme' indicates a system or a preparation in which the enzyme is attached on surface or confined/ localized within the semipermeable microcavities of solid polymeric supports (Marconi, 1984). In other words, the immobilized enzymes are physically localized in a certain region of space or converted from water soluble mobile state to a water insoluble immobile state (Martinek and Mozhaev, 1985, Klibanov, 1983) Immobilized enzymes may very

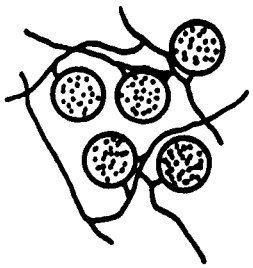
closely simulate the state of enzymatic proteins within the intracellular microenvironment of living cells and so they can provide a good model system for the study of *in vivo* behaviour and solving some basic problems in enzymology (Martinek and Mozhaev, 1985; Bickerstaff, 1982)

Table 1 1 lists important industrial applications of some enzymes Biosensors, based on immobilized enzymes or whole cells, are also finding wide spread applications in the analytical and biomedical fields (Suzuki and Karube, 1981; Guilbault, 1982; Scheper and Buckmann, 1990, Schelp *et al.*, 1991) and in the area of genetic engineering (Nilsson and Mosbach, 1981, Bulow and Mosbach, 1982, Winter *et al.*, 1982, Ulmer, 1983)

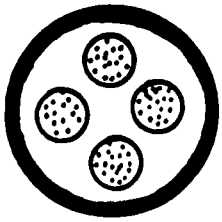
In the last three decades a very large number of methods of immobilizing enzymes have been developed and it is very difficult to identify an universally suitable method applicable to all enzymes The success of enzyme immobilization clearly depends on the choice of carrier and method of immobilization. Various organic and inorganic materials (Weetal, 1970; Mosbach, 1976, Wingard *et al.*, 1976, Van Beynum, 1980; Marconi, 1984) and even living cells have been used as supports for the enzyme immobilization (Synowiecki *et al.*, 1981; Uhm and Byun, 1984) The enzyme immobilization techniques can be broadly classified into the following categories (1) Adsorption, (2) Covalent attachment, (3) Chemical aggregation, (4) Entrapment and (5) Microencapsulation (Figure 1 1) Extensive literature is available on enzymes immobilized by various strategies which is beyond the scope of this review A short description of the immobilization techniques is however presented below



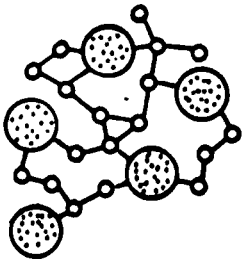
Adsorption



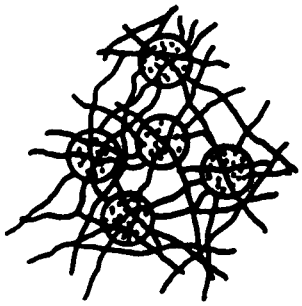
Covalent Binding



Microencapsulation



Aggregation



Entrapment

Table 1.1

Some enzymes of industrial and other applications

Enzymes	Applications
Amino acid acylase	Resolution of D, L amino acids
α-amylase	Treatment of wastewater from paper manufacturing
Asparaginase	As an anticancer agent
Catalase	Increasing shelf life of milk
β-Cellobiase	Hydrolysis of cellobiose to glucose
Cellulase	Hydroysis of cellulose into cellobiose and glucose
Dextranase	Removal of tooth plaques
β-galactosidase	Hydrolysis of lactose in milk or whey.
Glucoamylase	Conversion of starch to glucose.
Glucose isomerase	Conversion of glucose to fructose.
Glucose oxidase	Gluconic acid production and Glucose analysis in blood and urine.
Hyaluronidase	Hyrolysis of hyaluronic acid
Hydantoinase	Production of optically active amino acids from racemic amino acids
Invertase	Inversion of sucrose
Isoamylase	Production of maltose from starch
Keratinase	Modification of wool, hair and leather
Laccase	Drying of lacquer
Lipase	Flavouring of milk products and production of modified fats
Lysozyme	Treatment of certain ulcers, measles, multiple sclerosis and some skin diseases

Naringinase	Removal of bitter taste from citrus juices
Nitrilase	Production of acrylamide from acrylonitrile.
Papain	Beer chill proofing.
Pectinase	Fruit juice and wine clarification.
Penicillin amidase	Production of semisynthetic penicillin
Pepsin	Cheese making.
5'phosphodiesterase	Inosinic acid and guanylic acid production.
Proteases	*Protein hydrolysis*
Ribonuclease	Production of 5'-nucleotides from RNA
Streptokinase	Digestion of blood clots.

1. Adsorption

Adsorption is the fixation of an enzyme on the surface of a solid support that has not been specifically functionalized for covalent attachment. Adsorption is a complex process and may involve in the formation of various non covalent interactions, i e hydrogen bonding, hydrophobic and electrostatic interactions The reversibility of adsorption may be of practical advantage, since intact recovery of both enzyme and carrier may be possible Various supports including calcium phosphate gels, porous glass, carbon, silica, alumina, ion exchangers and even living cells have also been used for the immobilization of enzymes by adsorption (Levin *et al.*, 1979; Kitao *et al.*, 1981).

2. Covalent Attachment

This involves the activation of the support material for reaction with the side chain amino groups of protein or the use of a coupling reagent for linkage of the protein and support matrix Alternatively protein can also be activated prior to binding to the supports. The water insoluble supports may also be either organic polymers like agarose, cellulose, starch, dextrans, Sephadex, nylon and acrylamide alkylamine, etc. Inorganic supports like silica, alumina and carbon have been used for the insolubilization of enzymes. Covalent attachment is usually a permanent method of immobilization This minimizes the risk of enzyme desorption during operation but does not permit the reusability of the matrix.

3. Chemical Aggregation

Enzyme immobilization can be achieved by either chemical

aggregation/insolubilization with bifunctional or polyfunctional agents. These reagents are used for the insolubilization of active protein molecules or for binding these molecules onto specific suitable support

Among the numerous crosslinkers available glutaraldehyde, hexamethylene diisocyanate and 1,5-difluoro-2,4-dinitrobenzene are more widely used (Goldstein and Manecke, 1976). Some interesting reports on crosslinking of enzymes adsorbed onto supports are also available (Iqbal and Saleemuddin, 1983a; 1983b; Yamazaki, *et al.*, 1984). One advantage of chemical aggregation is that no carrier or support is added to the mass of immobilized material

4. Entrapment

Entrapment is achieved either by the formation of a crosslinked polymeric network around the enzyme molecule or the enzyme is placed inside a polymeric material and the polymeric chains are then crosslinked. This is followed usually by disruption of the polymeric mass into the desired particle size. The most commonly used polymeric system is polyacrylamide although alginate, silica, starch, polyamide and silicon rubber have been also used. Several reports describing the immobilization of enzymes by entrapment in polymeric matrices are also available (Lim and Sun, 1980; Sharma *et al.*, 1982; Lamberti and Sefton, 1983; Mattiasson, 1983). The entrapment procedures are generally not suitable for enzymes acting on large molecular weight substrates.

5. Microencapsulation

It may be achieved either by interfacial polymerization (a

chemical process) or by coacervation (a physical phenomenon). This method of immobilization is suitable for enzymes acting on low molecular weight substrates.

Immobilization of enzyme by entrapment in microcapsules was first reported by Chang (1964). These studies were subsequently continued by others (Rambourg *et al.*, 1980, Levy *et al.*, 1980). The significance of encapsulation is that enzymes remain chemically unmodified and hence catalytically active. Microencapsulation of enzymes provides large surface area for contact of substrate and catalyst but all within a relatively small volume. This method also allows the simultaneous immobilization of many enzymes in a single step.

GLYCOPROTEIN ENZYMES

Glycoproteins are characterized by the presence of covalently linked oligosaccharides and are widely distributed in animals, plants as well as in microorganisms. Although many biologically active components in living organism such as enzymes, hormones, toxins and carrier proteins are glycoproteinic difficulties in the study of the structure of the oligosaccharide moieties, their specific functions were ignored during the early years of protein research The significance of the glycoprotein glycosyl residues in a variety of *in vivo* functions is being increasingly realized (Goochee *et al.*, 1992)

Physiochemical functions of the carbohydrate moieties of several glycoproteins have been revealed through the use of the antibiotic tunicamycin (Tamura, 1982). Atleast in case of some enzymes the role of glycosyl residue on the activity, stability against denaturation and proteolysis has been established (Hu and Van, Huystee, 1989, Goochee

et al., 1992). The role of the carbohydrate moieties in signaling various cellular recognition phenomenon is also now recognized and a complete list of these works have been summarized by Tamura (1982).

1. Classification of sugar chains

The carbohydrate moieties of most glycoproteins are linked to the polypeptide backbone by either GalNAcα1-Ser (or Thr) linkage or GalNAc β1-Asn linkage. Sugar chains that fall into the former groups are also called mucin type or O-linked because they are found most abundantly in mucins. In contrast, sugar chain of the latter group were initially found more often in serum glycoproteins and are called serum type or N-linked. In recent years, the group designation "asparagine-linked" is more frequently used. The O-linked and N-linked sugar chains have quite different structural features. So far, no mannose has been found in O-linked sugar chains, while N-linked sugar chains always contain mannosyl residues Through studies of amino acid sequences of glycoproteins it was found that the asparagin residues bearing carbohydrate chains always occur in either an Asn-X-Ser or Asn-X-Thr sequence in which X represents one of the 20 amino acids other than proline. This suggested that these two tripeptides are recognized by the enzymes that is form of N-linked sugar chains (Bause and Hettkamp, 1979).

In contrast to N-linked sugar chains, no definite amino acid sequence is required for the formation of O-linked sugar chains The two types of major sugar chains of glycoproteins should therefore be considered quite distinct prosthetic groups Structures of typical N-linked and O-linked oligosaccharides are given in Figure 1 2 Numerous

Figure 1.2

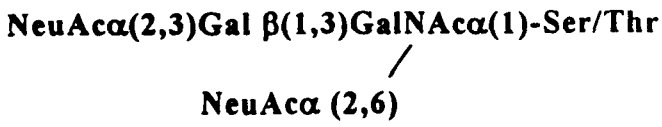
Some typical O-linked and N-linked oligosaccharides.

A. O-linked mammalian oligosaccharide

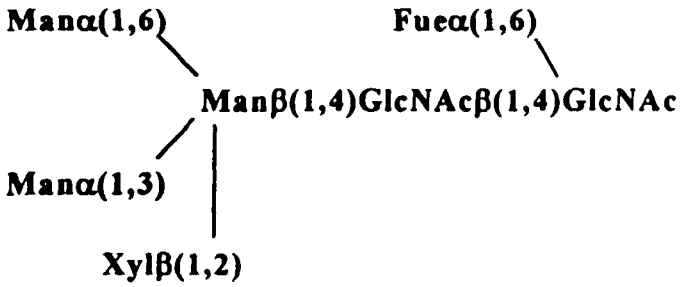
B. N-linked Plant enzyme laccase oligosaccharide

C. N-linked mammalian complex type oligosaccharide

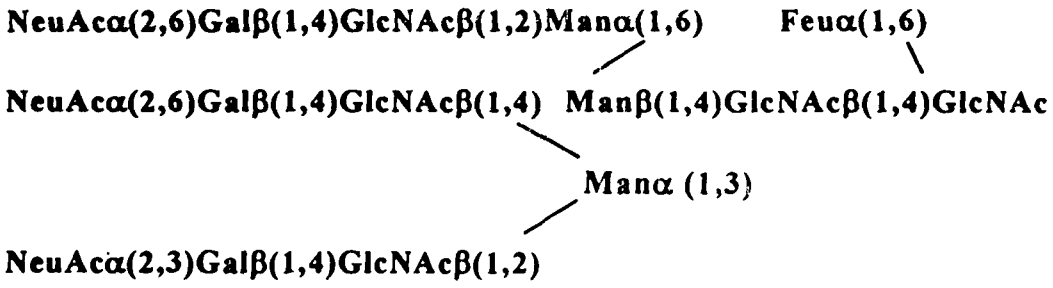
D. N-linked mammalian hybrid type oligosaccharide


NeuAcα(2,3)Gal β(1,3)GalNAcα(1)-Ser/Thr
NeuAcα (2,6)

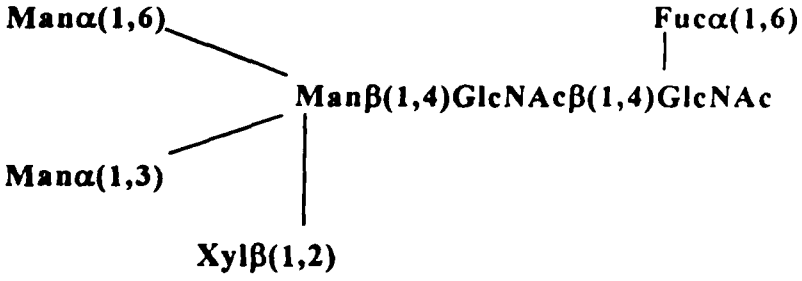
A


Manα(1,6)
Fueα(1,6)
Manβ(1,4)GlcNAcβ(1,4)GlcNAc
Manα(1,3)
Xylβ(1,2)

B


NeuAcα(2,6)Galβ(1,4)GlcNAcβ(1,2)Manα(1,6)
Feuα(1,6)
NeuAcα(2,6)Galβ(1,4)GlcNAcβ(1,4)
Manβ(1,4)GlcNAcβ(1,4)GlcNAc
Manα (1,3)
NeuAcα(2,3)Galβ(1,4)GlcNAcβ(1,2)

C


Manα(1,6)
Fucα(1,6)
Manβ(1,4)GlcNAcβ(1,4)GlcNAc
Manα(1,3)
Xylβ(1,2)

D

variation of both type of chain have been however reported Several sugar chains that do not fall into either of these two groups also occur in some glycoproteins, but their distribution is very limited Galactosylhydroxylysine is found as linkage structure of collagens (Bornstein and Traub, 1979), while mannosyl-serine occurs in mannans that compose the yeast cell wall (Ballou, 1976), and also in some microbial enzymes (Rosenthal and Nordin, 1975) Some plant glycoproteins such as extensins and potato lectin contain oligoarabinosyl residues linked to hydroxyproline and serine groups (Lamport, 1967; Allen *et al.*, 1978)

2. Role of carbohydrate moieties of glycoenzymes

The biological functions of several glycoproteins are well established but the role that their carbohydrate plays in these functions is, for the most part, unclear. Potential functions of the glycosyl moieties include stabilization of tertiary structure (Olden *et al.*, 1985, Paulson, 1989; West, 1986), protection from proteolytic degradation (Olden *et al.*, 1985, Paulson, 1989), transport of macromolecules (Eylar, 1965), organization of macromolecules into oligomeric forms (Morgan *et al.*, 1970) and action as sorting signals for directing proteins to specific cellular organelles and tissues (Paulson, 1989) The carbohydrate portion of IgG may be implicated in the recognition of antibody of Fc receptor and complement (Koide *et al.*, 1977) Nonetheless, it has been difficult to uneaquivocally demonstrate one or more of the functions experimentally Thus, no detectable ellipticity differences between glycosylated and non glycosylated ribonuclease β could be found (Puett, 1973) Frederick *et al.*, (1978) have demonstrated that removal of carbohydrate moiety from a variety of

glycoproteins such as deoxyribonuclease, ribonuclease β, invertase, carboxypeptidase Y and penicillium nuclease P_1 has no apparent effect on the enzymatic activities or on CD spectra of these glycoproteins (Tarentino *et al.*, 1974; Trimble and Maley, 1977). Frederick *et al.*, (1978) have shown that the carbohydrate moiety of external invertase from *Saccharomyces cerevisiae* promotes the correct refolding of the unfolded enzyme *in vitro*, suggesting that carbohydrate chains may, in some instances aid or direct the folding of the nascent polypeptide chains. Nagai and Yamaguchi (1993) investigated the role of the high-mannose oligosaccharide chains in the folding and assembly of soybean lectin polypeptide. Soybean lectin, dissociated into subunits and completely denatured in 6.0M guanidine hydrochloride, was quantitatively reconstituted to the active tetrameric structure by simple dilution. On the other hand, the deglycosylated subunits lacked the ability to regain neither tetrameric structure nor biological activity, suggesting essential role of the high-mannose oligosaccharide chains in the correct folding and assembly of glycopeptides.

Moyle *et al.* (1975) showed that removal of the sugar chains of human gonadotropin (hCG) by sequential exoglucosidase digestion gradually reduced the ability of the hormone to stimulate the production of cAMP and testosterone by rat Leydig cells. This was followed by several investigations on the effect of enzymatic or chemical deglycosylation on the biological activity of hCG (Kalyan *et al.*, 1982; Goverman *et al.*, 1982; Chem *et al.*, 1982). Removal of sialic acid from hCG enhanced the binding of the hormone to receptor on the target cell, but its hormonal activity was lowered by 50%. Removal of the remaining part of its sugar chain further enhanced the binding of the hormone to the receptor but stripped it completely of its hormonal

activity, indicating the role of glycosyl residues in the full expression of the hormonal activity of hCG

Piesecki and Alhadeff (1992) have shown that removal of carbohydrate from α-L-fucosidase does not affect its catalytic activity, Km for synthetic substrate, recognition and rate of hydrolysis of three natural substrates or gross conformation. However, carbohydrate removal does lead to decrease in enzymatic activity in acidic pH, a neutral shift in pH optimum and thermostability.

IMMOBILIZATION OF GLYCOENZYMES

Among the various methods employed for the immobilization of enzyme, those involving coupling to solid supports are most widely used (Ichijo *et al.*, 1982; Ohmiya *et al.*, 1978). Enzymes are coupled to the insoluble matrices through amino, carboxyl or phenolic or other groups of amino acid side chains. Covalent coupling of glycoenzymes to solid supports is, however, manifest with problems due to masking of the reactive amino acid side chains by oligosaccharides (Liberatore, *et al.*, 1976; Pollak *et al.*, 1978). The carbohydrate spikes project from the surface of the glycoproteins and shield the reactive functional groups (Hsiao and Royer, 1979). Liberatore *et al* (1976), attempted with very little success, to immobilize carboxypeptidase Y, a glycoenzyme, by covalent coupling on hexamethylene diamine agarose Attention was, therefore, directed towards strategies in which carbohydrates form the point of attachment between the enzyme and support (Woodward and Wiseman, 1978) However, it was possible to increase the immobilization yield of several glycoenzymes by introducing reactive groups in carbohydrate chains followed by their coupling to activated supports (Zaborsky and Ogletree, 1974; Hsiao

and Royer, 1979, Valentova *et al.*, 1981, Marek *et al.*, 1984)

Hsiao and Royer (1979) made a study of several glycoenzymes including peroxidase, carboxypeptidase and glucose oxidase, immobilized through their carbohydrate chains The carbohydrate residues of the enzymes were oxidized with periodate to generate functional aldehyde groups. Treatment with amines (ethylenediamine or glycyltyrosine) and borohydride provided groups through which the enzyme could be connected to the support Ethylenediamine treated enzymes were coupled to an amino caproate adducts of CL Sepharose via N-hydroxy succinimide ester or to cyanogen bromide activated Sepharose. Coupling yields obtained in the range of 37-50% The insoluble derivatives of glycoenzymes showed good activity (71-83% of native). Stability studies on the immobilized aminoalkyl enzymes were encouraging and the immobilized forms of glucoamylase, glucose oxidase and carboxypeptidase retained full activity after six assays in stirred suspension.

GLYCOENZYMES IMMOBILIZATION USING CONCANAVALIN A

Con A, originally isolated by Sumner in 1919 from the Jack bean *Canavalia ensiformis* is a plant lectin. Sumner and Howell (1936) subsequently identified the hemagglutination property of Con A Goldstein *et al.* (1965) have investigated the specificity of Con A for carbohydrates in detail The lectin is specific towards α-mannopyranoside and α-glucopyranoside and α-N-acetyl-D- glucosamine (Goldstein *et al.*, 1965) Specific saccharide binding property of Con A has been widely utilized in the purification, characterization and sequencing of polysaccharides, glycopeptides and glycoproteins

(Nicolson, 1976).

Con A has been employed as a tool for a variety of studies (Nicolson, 1974; Yahara and Edelman, 1973). More recently, several investigators studied the usefulness of Con A as an effective ligand in the immobilization of glycoenzymes (Saleemuddin and Husain, 1991).

Most of the enzymes are currently being used in industry are derived from moulds and are glycosylated to varying extent. These include invertase, HRP, glucose oxidase, cellulose and amyloglucosidase, etc. Immobilization of glycoenzymes on Con A support received remarkable attention due to retention of high activity by the lectin associated enzymes. Immobilization of Con A matrices also appears to offer several distinct advantages including high yield of immobilization and enhanced resistance to various forms of inactivation etc. (Iqbal and Saleemuddin, 1983b, Turkova *et al.*, 1986)

Immobilization of glycoenzymes on Con A support has been generally achieved by specific adsorption sometimes followed by covalent coupling. The strategies used for the preparation of immobilized glycoenzymes using Con A are (i) immobilization of glycoenzymes on matrices precoupled with Con A, and (ii) preparation of insoluble Con A-glycoenzymes flocculates. A number of glycoenzymes have been immobilized by these techniques and their stability and other properties investigated (Saleemuddin and Husain, 1991).

Sulkowski and Laskowski (1974) demonstrated for the first time the use of Con A matrix bound exonuclease in the continous hydrolysis of polynucleotides. The preparation was highly efficient in the hydrolysis of poly A and ApAp The immobilized preparation was also stable to storage for over one month at pH 8.5 at room temperature

Woodward and Wiseman (1978) achieved high immobilization

yields by binding yeast invertase on Con A-agarose. The Km of immobilized invertase was similar to that of the native enzyme, and preparation showed significant broadening of the pH activity profile and marked improvement in stability at 65°C. Iqbal and Saleemuddin (1985) reported a marked enhancement in stability of yeast invertase against heat and chemical denaturants after immobilization on Con A-Sepharose. The Con A-Sepharose bound invertase retained markedly by higher activity than the soluble enzyme at 60°C or in presence of 3.0M urea. The stabilization against heat and urea was further improved by crosslinking the preparation with glutaraldehyde. A laboratory scale column (2.0 x 10cm) of the crosslinked preparation was operated by the authors for over 60 days without significant decrease in invertase activity. A solution of 1.0M sucrose passed through the column at flow rate of 100 ml h^{-1} was hydrolyzed over 80% at 30°C during the hydrolysis.

Iqbal and Saleemuddin (1983b) investigated the activity and stability of various invertase and glucose oxidase preparations immobilized on Sepharose matrices precoupled with either 'low' and 'high' Con A concentration. The high-lectin bound preparations, exhibited relatively greater increase in stability as compared to those immobilized on low-lectin matrices.

In a more recent study, Reddy and Shanker (1989a) observed that *Asperqillus oryzae* S_1-nuclease retained high activity when immobilized on Sepharose matrix precoupled with 1.0mg Con A ml^{-1}, very low activity was, however, expressed when the enzyme was bound on Sepharose matrix containing 8.0mg Con A ml^{-1}, unless glucose was included in the reaction mixture during the coupling process. The low-lectin bound S_1-nuclease exhibited unaltered pH activity profile but was markedly more stable to heat and urea denaturation than the soluble

enzyme These authors also immobilized partially purified RNase T_2 from *Aspergillus oryzae* on Sepharose matrix precoupled with varying amount of Con A. The immobilized enzyme preparations exhibited low Km, enhanced thermal stability, and higher resistance to metal ions (Reddy & Shanker, 1989b).

In an attempt to cut down the cost of the Con A matrix, Husain and Saleemuddin (1989) incubated Sephadex G-50 with crude Jack bean extract, instead of pure Con A, and used the preparation for the immobilization of various glycoenzymes, including cellulase, cellobiase, invertase, amyloglucosidase and glucose oxidase. The enzymes were immobilized in high yields. As compared to the respective soluble enzymes, the immobilized preparation of cellobiase and glucose oxidase exhibited high stability against heat denaturation and trypsin inactivation especially after crosslinking with glutaraldehyde.

Complexing with Con A does not seem to interfere with the expression of catalytic activity of glycoenzymes in most cases, presumably due to the distant location of the oligosaccharide chains from the enzyme active site. The insoluble Con A-arylsulfatase complex prepared thus exhibited high enzyme activity and a striking increase in stability against heat and chemical denaturation (Ahmed *et al.*, 1973). The sheep brain lysosomal hydrolase arylsulfatase A, acid phosphatase, β-N-acetyl hexosaminidase, β-D-galactosidase, and β-glucouronidase, formed enzymatically highly active precipitates with Con A. (Bishayee and Bacchawat, 1974). Various glycoenzymes form insoluble complexes with Con A at different Con A-enzyme ratio's. Glucose oxidase, amyloglucosidase (Husain *et al*, 1985; Husain and Saleemuddin, 1986) and plasma amino oxidase (Ishizaki and Yasunobu, 1980) precipitate at Con A-glycoenzymes ratio's of less than one, isolubilization of yeast

invertase requires a molar ratio of 12. (Husain *et al.*, 1985). Glucose oxidase retained essentially complete activity, while invertase and amyloglucosidase exhibited only 70% of the original activity in the complex form. The Con A-invertase complex exhibited a dramatic increase in stability over the soluble enzyme which was further improved by crosslinking with 0.5% glutaraldehyde. In a subsequent study, Husain and Saleemuddin (1986) reported an inexpensive procedure for the preparation of Con A glycoenzymes adducts using crude Jack bean extract. Con A support thus appear to have a remarkable potential in the immobilization of glycoenzymes for a variety of applications.

HORSERADISH PEROXIDASE

Horseradish peroxidase (HRP, EC. 1.11.1.7) occurs in nearly all plant and in some animal cells. Highest concentration has been, however, reported in the sap of the fig trees while the horseradish roots are also quite rich in peroxidase. Many Peroxidases occur in plant cell walls (Mader *et al.*, 1975) where they participate in lignin synthesis (Goldberg *et al.*, 1983).

The specificities of all peroxidases for their substrates are high, only hydrogen peroxide and mono substituted alkali peroxides, like methyl and ethyl derivatives act as substrates (Chance, 1949). Extensive lists of these compounds were published by Kastle & Porch (1908).

Complete primary structure of cationic HRP has been established (Welinder, 1976). The cationic HRP consist of a single polypeptide chain of 308 amino acid, hemin prosthetic group and two moles calcium per mole enzyme. Each molecule has four disulfide bridges and eight neutral carbohydrate side chains. The molecular weight of

cationic horseradish peroxidase ranges between 40,000 and 45,000 (Welinder, 1976).

The carbohydrate moieties of HRP constitute about 18% of its total weight (Welinder, 1976). The eight neutral carbohydrate side chains are exposed on the surface of the enzyme (Welinder, 1985). Some peroxidases bind to Concanavalin A (Con A) while others do not (Van Huystee, 1987). However, if those that do not lack glycosylation is not clear. Atleast six glycosidic peptides of HRP show affinity for Con A (Derbyshire, 1973). The size of their N-linked (asparagine linked) oligosaccharide chains ranges from 1600 to 3000 with mannose and glucosamine as their prominent sugars (Clarke and Shannon, 1976).

It has been known for a long time that many spectral and catalytic properties are shared by plant peroxidases, myoglobins and Hemoglobin (Keilin and Hartee, 1951) suggesting the presence of a similar heme prosthetic groups.

Horseradish peroxidase is a metalloprotein, in which calcium contributes to the structural stability of the protein (Haschke & Friedhoff, 1978). Calcium is normally an integral part of peroxidase molecule (Welinder, 1985). Native HRP contains two moles of calcium per mole of enzyme (Haschke and Friedhoff, 1978). The bound calcium may be removed by incubation of peroxidase with quanidine - HCl and EDTA. However, EDTA alone removed the calcium only very slowly (Haschke & Friedhoff, 1978). The activity of HRP also falls after removal of calcium and it is restored upon reconstitution with calcium (Haschke and Friedhoff, 1978)

Isoenzymes of peroxidase were originally detected by Theorell (1942) in horseradish roots. It is now recognized that peroxidase

isoenzymes are present throughout the plant kingdom (Morita and Kameda. 1961, Hosoya. 1960) While all peroxidase isoenzymes appear to catalyze the same reaction, individual isoenzyme may differ markedly in physiochemical and Kinetic properties (Hosoya, 1960; Morita *et al.*, 1962).

Seven peroxidase isoenzymes were isolated from horseradish roots and purified to homogeneity as ascertained by ion exchange chromatograhy and polyacrylamide disk electrophoresis (Shannon *et al.*, 1966). These isoenzymes were broadly classified into anionic, neutral and cationic forms. No interconversion among the isoenzymes was detected.

1. Immobilized HRP

Few investigators have immobilized HRP on a variety of supports and investigated the properties. Berezin *et al.* (1977) immobilized the HRP after periodate oxidation of its glycosyl residues on AH-Sepharose 4B. The Sepharose bound enzyme exhibited unaltered Km value for hydrogen peroxide while that for O-diansidine hydrochoride decreased by about 20%. Thermal stability of the immobilized HRP was greater than the respective soluble enzymes. HRP was also immobilized on various polysaccharide matrices such as Sepharose, Sephadex G-100, starch and cellulose by graft copolymerization reaction (Anqiuro *et al.*, 1980) to yield preparations with superior operation and storage stability.

Anqiuro *et al.*, (1987) immobilized HRP on agarose by a photochemically initiated graft copolymerization reaction, carried out at room temperature. Hexhydro-1,3,5 triacryloyl-s-triazine (HTST) used as vinylating reagent. The immobilized preparation thus obtained

showed good mechanical and enzymatic stability at elevated temperature. However, the immobilization yield was not satisfactory (Cremonesi and Anqiuro, 1983). The drawbacks were overcome and the immobilization yield increased from 15 to 30% by carrying out the immobilization at 35°C instead of at 20°C. (Angiuro and Lalla, 1985). The polymer appeared to have an effective role and better stability properties were generally obtained when the concentration of polymer was high.

Sandwick and Schray (1988) immobilized the HRP by the process of adsorption on Sepharose and investigated the conformation of enzyme bound to Sepharose. The adsorbed enzyme exhibited altered structural conformation at relative low concentration while at high concentration of enzyme such conformational alterations were not observed when more enzyme was loaded in the support.

HRP was also immobilized on segmented polyetheurethene SPEU films via radiations grafting by Hongfer *et al.*, (1988). In this case acrylic acid or acrylamide was grafted via radiation onto the film through chemical binding.

INVERTASE

β-fructofuranosidase; β-D-fructofuranoside fructo-hydrolase; (EC 3.2.1.26) more commonly known as invertase, was discovered in the *Baker's yeast* by Berthlot (1860). Invertase hydrolyzes compounds with any substituted β-D fructofuranosyl residues, such as sucrose, raffinose and methyl β-D-fructofuranoside.

Yeast invertase exists in two distinct forms, the non glycosylated internal and the heavily glycosylated external enzyme (Gascon *et al.*, 1968). The external form is the predominant in *Baker's yeast* and

occurs mainly in the periplasmic space. It is a glycoprotein containing about 50% (w/w) of its mass as carbohydrate in the form of high mannose oligosaccharide chains (Trimble and Maley, 1977). The 270,000 of external invertase (Neumann and Lampen, 1967) consists of two 60,000 Mr polypeptide subunits, the residual mass being carbohydrate (Trimble and Maley, 1977). The carbohydrate moieties do not appeared to be essential for catalytic activity of invertase (Smith and Ballou, 1974; Tarentino *et al.*, 1974).

External and internal invertase are identical in their polypeptide composition (Chu *et al.*, 1985; William *et al.*, 1985). The carbohydrate moieties of the external invertase however appear to enhance the renaturation efficiency of the enzyme and stabilize its activity under a variety of conditions (Chu *et al.*, 1978). The glycosyl residues also enhanced resistance of the polypeptide backbone to proteolysis (Brown *et al.*, 1979). Chu *et al.* (1983) have shown that the carbohydrate components of invertase facilitate subunit interactions and formation of active oligomers (i.e. mostly octamer, hexamer and tetramer) and the enzyme dissociate into dimers on deglycosylation.

1. Immobilized Invertase

It is of interest to note that the first enzyme to be immobilized appears to be invertase (Nelson and Griffin, 1916). A large number of reports describe the immobilization of invertase on a variety of supports. Invertase has been immobilized by adsorption on bentonite followed by crosslinking with cyanuric chloride (Monsan and Durand, 1971), adsorption on DEAE-cellulose (Suzuki *et al.*, 1966), and covalently coupled to polyaminostyrene tubes (Filippusson and Hornby, 1970) and to porous glass (Mason and Weetal, 1972). Invertase immobilized on

bentonite exhibited enhanced thermal stability (Monsan and Durand, 1971). High storage stability was achieved when invertase was immobilized by the process of adsorption on the DEAE-cellulose matrix (Usami *et al.*, 1971).

Invertase immobilized by microencapsulation in the polyamide microcapsules was used for hydrolysis of the accumulated sucrose in luman that prompts intense diarrhoea, amplified further by microbial fermentation (Levy *et al.*, 1980, Rambourg *et al.*, 1982). Invertase from *Saccharomyces cerevisiae* was also immobilized by adsorption or covalent coupling on amino polystyrene using glutaraldehyde and carbodiimide (Panlyukonis *et al.*, 1980). Hen Egg white was used as support for the immobilization of invertase by D'souza and Nadkarni (1981), who observed that the immobilized enzyme could be repeatedly used for sucrose hydrolysis in batch process. Krill Chitin was shown to be a useful support for the immobilization of invertase (Synowiecki *et al.*, 1981). Leela and Prabhakara (1982) obtained a better recovery of immobilized invertase with the molecular sieve 4A using the metal link method. Other supports used for the immobilization of invertase include Sepharose and modified hydrophobic cellulose derivatives (Choi *et al.*, 1982). Uedaira *et al.* (1984) have demonstrated the thermostability of invertase immobilized in a film of two kinds of photocrosslinked polyvinyl alcohol, the denaturaion temperature of immobilized enzyme was raised and denaturation enthalpy was decreased. Glycosidic component of invertase was treated with periodate and the enzyme covalently bound onto three types of modified solid supports, of which invertase bound to the styrene divinyl benzene copolymer was found most stable (Marek *et al.*, 1984).

Calcium alginate entrapped invertase also showed no significant

decrease in sucrose hydrolysis for over 6 days (Uhm *et al.*, 1984). Yamazaki *et al.* (1984) described the preparation of a column containing invertase bound to cloth segments. The column exhibited the good flow properties and efficient sucrose hydrolysis for over three months. Invertase immobilized onto corn grits was utilized by Monsan and Combes (1984) for the hydrolysis of highly concentrated sucrose solution in the continuous stirred tank reactor and more efficiently in plug flow reactor.

AMYLOGLUCOSIDASE

Amyloglucosidase (α-1,4-D-glucan glucohydrolase EC 3.2.1.3) is an extracellular enzyme produced by *Aspergillus niger* and *Rhizopus sp* It acts on α-1,3, α-1,4 and α-1,6 and glucosidic bonds (Pazur and Kleppe, 1962; Thoma *et al.*, 1971). All the amyloglucosidases so far studied are glycoproteinic (Ohga *et al.*, 1966, Manjunath *et al.*, 1983). Amyloglucosidase isolated from *Rhizopus sp.* have three different isozymes forms and that have the same C-terminal amino acid sequences but differ in that at the N-terminal end (Takahashi *et al.*, 1982). Two different types of amyloglucosidases have been isolated from *Aspergillus niger*. Among these the major form has 18% carbohydrate and an pH optimum of 4.8. Removal of carbohydrate moieties by treatment with sodium (meta) periodate or with anhydrous hydrogen fluoride resulted in decrease thermal stability, indicating that carbohydrate groups are necessary for stability of the native conformation but not for catalytic activity or antigenecity of the enzyme (Shenoy *et al.*, 1985). Fungal amyloglucosidases hydrolyze starch, maltose and other carbohydrates having α-1,3 and α-1,4, glycosidic linkages. The rate of reaction is mainly dependent on the chain length of the substrate and the Km value

markedly decrease with increasing chain length (Ono *et al.*, 1964)

1. Immobilized Amyloglucosidase

An enzyme of immense industrial significance amyloglucosidase has been subjected to immobilization by large number of procedures. These include those based on adsorption, covalent coupling to solid supports, encapsulation etc. The work has been extensively reviewed (Olson and Korus, 1977; Pitcher, 1980; Shankar *et al.*, 1993).

Usami and Taketomi (1965) immobilized amyloglucosidase on a matrix of diatomaceous earth and acid clay and obtained immobilized enzyme having high activity, however, the immobilized preparation was not stable and the enzyme leached out in presence of 2% starch. Amyloglucosidase has been immobilized by entrapment in polyacrylamide gels (Mariyama *et al.*, 1980), binding onto DEAE-cellulose (Jach and Sugier, 1983) and coupling to cyanogen bromide activated Dextran (Wongkhalaung *et al.*, 1985). The preparations exhibited varying degree of stabilization against various forms of denaturation. Amyloglucosidase immobilized on non-porous cationic glass beads expressed over 80% of total enzyme activity (Wasserman *et al.*, 1982) Amyloglucosidase immobilized on acrylic ion exchangers of the phonilex type displayed high specific activity (Maxim *et al.*, 1986), Chibata (1978) has demonstrated the small scale production of glucose using amyloglucosidase immobilized on 2-amino-4,6 dichloro-5-triazine activated DEAE-cellulose. Dextran coupled amyloglucosidase entrapped in calcium alginate was shown to be effective in the conversion of oligodextrins into glucose during a two months period in a packed bed reactor (Svensson and Ottessen, 1981)

Amyloglucosidase immobilized on zeolite was successfully used

in the conversion of liquified starch mass into high glucose syrup Effective hydrolysis of maltodextrin was achieved in a fluidized bed reactor with the help of amyloglucosidase grafted onto corn stover (Vallat *et al.*, 1986).

GLUCOSE OXIDASE

Glucose oxidase (B-D-glucose:Oxygen 1-oxidoreductase; EC 1.1.3.4) a glycoprotein having molecular weight of 150,000-180,000. It contains two moles of FAD per mole of enzyme and 16% carbohydrate which include mannose, galactose and glucosamine, probably as the N-acetyl derivatives. Glucose oxidase is relatively rich in aspartic acid, glutamic acid, glycine and alanine but relatively low concentration of cysteine, methionine and tryptophan. (Pazur *et al*, 1965; Hayashi and Nakamura, 1981).

1. Immobilized Glucose Oxidase

Glucose oxidase has been stabilized by immobilization by several investigators. An increase in stability was observed when glucose oxidase was immobilized on alumina, hydroxyapatite and porous glass (Weetal, 1970). Stabilization of glucose oxidase immobilized on six porous inorganic supports was observed at all pH values investigated (Bouin and Hultin, 1982). A glucose oxidase preparation of high thermostability was obtained by physical entrapment of the enzyme in polyacrylamide gels (Rao *et al.*, 1981). Immobilization of glucose oxidase and catalase on activated 13X Zeolites also yield stable preparations (Pifferi *et al.*, 1982). A glucose oxidase preparation immobilized in collagen membrane, showed alterations in Km value but no change in pH or temperature optimum (Gozia *et al.*, 1983)

Glucose oxidase adsorbed on zinc particles and entrapped in polyacrylamide gels exhibited high specific activity and stability (Tikhonova *et al.*, 1985). Wehnert *et al.* (1985) have shown that Eupergit C has a three times higher specific glucose oxidase binding capacity than CPG-10. Alberti and Klibanov (1982) used immobilized glucose oxidase for the preparative production of hydroquinone from benzoquinone. Benzoquinone replaced O_2 as an electron acceptor in the oxidation of β-D-glucose and hydroquinone could be produced in nearly 100% yield. Gluconic acid and fructose was also continuously produced using polymethyl methacrylate microcapsules containing glucose oxidase and invertase (Obata *et al.*, 1983).

Chapter II

EXPERIMENTAL

MATERIALS

The chemicals used in the present study were obtained from various sources as shown below

Chemical	Source
Acrylamide	Sigma Chem Co , USA
Ammonium sulphate	May and Baker, England
Acetic acid	E Merck, India
Amyloglucosidase	Sigma Chem Co , USA
Bovine Serum Albumin	Sigma Chem Co , USA
Bromophenol blue	B D H Poole, England
Calcium chloride	Sisco Res Lab , India
Carboxymethyl cellulose	B D H Poole, England
Concanavalin A	Sigma Chem Co , USA
Coomassie brilliant blue R-250	Sigma Chem Co , USA
Cyanogen bromide	Sisco Res Lab , India
D-Glucose	BDH, India
Dithiothretol	Sisco Res Lab , India
Ethanolamine	Sisco Res Lab , India
Ethylenediamine tetra acetic acid (EDTA)	Sisco Res Lab , India
Ethylenediamine	Sisco Res Lab , India
Ethylene glycol	Sisco Res Lab , India
Folin's Reagent	Sisco Res Lab , India
Glucose oxidase (*Aspergillus niger*)	Sigma Chem Co , USA

Glutaraldehyde	Kock Light, England
Hydrogen peroxide	E Merck, India
Isopropyl alcohol	E. Merck, India
Invertase (*Baker's yeast*)	Sigma Chem. Co., USA
Methyl α-D mannopyranoside	Sigma Chem. Co., USA
Methyl Cellosolve	Sisco Res. Lab., India
Ninhydrin	B.D.H. Poole, England
N,N'- methylene bisacrylamide	Sigma Chem. Co., USA
O-diansidine-HCl	CSIR Center for biochemicals, India
Peroxidase	Sigma Chem. Co., USA
Phenol	Loba Chem. Co., India
Sepharose-4B	Sigma Chem. Co., USA
Seralose-4B	Sisco Res. Lab., India
Sodium acetate	E. Merck, India
Sodium Lauryl sulphate	Sigma Chem. Co., USA
Sodium borohydrate	B.D.H. England
Sodium (meta) periodate	Loba Chem. Co., India
Sucrose	Sisco Res. Lab., India
Tris (hydroxymethyl aminomethane)	Sigma Chem. Co., U.S.A.
Urea	E. Merck, India

METHODS

I CHEMICAL MODIFICATIONS OF GLYCOENZYMES

A. Peroxidation of Glycoenzymes

Appropriate quantity of HRP, invertase, glucose oxidase and amyloglucosidase was dissolved as indicated in either 50mM sodium acetate buffer, pH 5.0 or sodium phosphate buffer pH 6.0-7.0 and mixed with 2mM or 10mM sodium (meta) periodate. The mixture obtained was continuously stirred at 4°C for 30-120 min. in dark. Unreacted periodate was then inactivated by 16% ethylene glycol.

B. Preparation of Ethylenediamine/Ethanolamine enzyme adducts

To appropriate aliquots of the periodate treated glycoenzyme was added ethylenediamine or ethanolamine, pH 8.0 to a final concentration of 0.1M in a total volume of 2.5 ml. This was followed by the addition of 0.1M $NaBH_4$ in 0.1M NaOH for 2.5 h at 4°C. The unreacted ethylenediamine or ethanolamine was removed by extensive dialysis either against 0.1M sodium acetate buffer, pH 5.0 or sodium phosphate buffer, pH 7.0 (Hsiao and Royer, 1979).

II. ENZYME IMMOBILIZATION AND RELATED PROCEDURES

A. Immobilization of Sepharose-4B/Seralose-4B

Sepharose 4B or Seralose 4B (a product comparable with Sepharose 4B, in properties produced by an Indian manufacturer (Sisco Res. Lab.) was activated as described by Porath *et al.* (1967). Five grams of Sepharose/Seralose was washed thoroughly with distilled water using a glass sintered funnel. The gel was sucked dry and suspended

in 5.0 ml distilled water and 5.0 ml 2.0M sodium carbonate and mixed thoroughly. 1.0 g cyanogen bromide dissolved in 1 0 ml acetonitrile was added to the beaker containing Sepharose /Seralose and mixed thoroughly at 4°C for 10 min. The whole mass was then transferred immediately to a glass sintered funnel and washed thoroughly with 0.1M bicarbonate buffer, pH 8.5, distilled water and once again with the buffer. The washed activated Sepharose/Seralose was dried and resuspended in 5.0 ml of 0.1M bicarbonate buffer, pH 8 5

Required amount of Con A (3-6 mg/ml) was dissolved in the appropriate buffer and stirred with cyanogen bromide activated Sepharose/Seralose for 24 h at 4°C. The Sepharose matrix with bound Con A was separated by centrifugation and protein in the supernatant was quantitated in order to calculate the amount of Con A immobilized. The Con A bound matrix was thoroughly washed with 0.1M sodium bicarbonate buffer, pH 8.5 containing 1.0 M NaCl 10.0 ml of this washed suspension was treated with 0.1 ml 98% ethanolamine for 2 h at 4°C. The Con A matrix was washed successively with 0.1M sodium bicarbonate, buffer, pH 8.5 containing 1.0 M NaCl, distilled water and finally with buffer. Before using, the Con A bound Sepharose/Seralose was activated using the procedure described by Yariv *et al.* (1968). Glycoenzymes dissolved in 0.1M buffers of pH 5-7 were stirred with Con A-Sepharose/Seralose at 4°C for 12 h. An aliquot of supernatant was retained to determine the extent of enzyme immobilization. The immobilized enzymes were thoroughly washed with buffers and finally suspended in working buffer.

The procedure followed for the immobilization of ethylenediamine and ethanolamine adducts of enzymes was essentially same as discussed above except that instead of the native enzyme, ethylenediamine or

ethanolamine treated preparations were used.

B. Crosslinking of Con A-Sepharose/Seralose-Glycoenzyme preparations

In order to obtain immobilized crosslinked preparations, the Con A Sepharose/Seralose bound HRP, invertase, Glucose oxidase and amyloglucosidase preparations were treated with 0.1% glutaraldehyde for 2 h at 4°C. The matrices were subsequently washed thoroughly and resuspended in appropriate buffers.

C. Determination of Covalent Coupling

Preparations containing native or periodate treated glycoenzymes bound to Con A Sepharose/Seralose were incubated with 0.5M methyl α-D-mannopyranoside for 12 h at 4°C with slow stirring. Aliquots of supernatant and washings taken out for the determination of the released enzyme activity. The matrix was thoroughly washed and resuspended in working buffers.

D. Effectiveness Factor, η values

The η values of the immobilized preparations represent the ratios of actual and theoretical activity of the immobilized enzyme (Muller and Zwing, 1982). Actual activity of the enzyme was determined by assaying an appropriate aliquot of the immobilized enzyme. The amount of immobilized enzyme (theoretical activity) was evaluated by subtracting the soluble enzyme activity remaining after immobilization from that added for immobilization.

III. COLUMN CHROMATOGRAPHY

A. Isolation of HRP isoenzymes

The anionic and cationic isoenzymes of HRP were isolated from the commercial preparation using ionexchange chromatography as described by Shannon *et al.* (1966).

B. CM-Cellulose Chromatography

Five grams of CM-cellulose was suspended in appropriate volumes of 5mM sodium acetate buffer, pH 4.4 to obtain homogenous slurry and poured into a column (20 x 1.8 cm). After allowing sufficient time to settle down, the flow rate of the column was adjusted. The crude HRP dissolved in 5mM sodium acetate buffer, pH 4.4 was transferred to the column and washed thoroughly, and then eluted with a linear gradient of 5-100mM sodium acetate buffer, pH 4.4. Just before the eluent was exhausted, a second elution system consisting of 100-250mM sodium acetate buffer, pH 4.4 was introduced into the system. Three millilitre fractions were collected and analyzed for peroxidase activity and protein (by absorption at 460 and 280 nm respectively). Anionic form of HRP was not retained on the column.

C. DEAE-Cellulose Chromatography

Appropriate amount of DEAE-cellulose was suspended with 15 volumes of 0.5N HCl for 30 min. and washed thoroughly using a Buchner funnel till the pH of the filtrate was approximately 4.0. The ion exchanger was then treated with 15 volume of 0.5N sodium hydroxide for 30 min and washing continued till the pH of the filtrate approached neutrality. The DEAE-cellulose was then resuspended in 5mM Tris HCl buffer, pH 8.4 to get a homogenous slurry. The Fine particles were

removed and poured into a column (20 x 1 8 cm)

The fraction containing HRP not retained on the CM-cellulose column was dialyzed against the 5mM Tris-HCl buffer, pH 8 4 and then transferred to a DEAE-cellulose column All the enzyme activity and protein of anionic fraction was retained The column was eluted with a linear gradient of 100 ml of 5mM Tris-HCl, buffer, pH 8 4 and 100 ml of 5mM-Tris HCl Buffer pH 8.4 containing 0 1M NaCl Three millilitre fractions were collected and analyzed for activity and protein

IV. GEL ELECTROPHORESIS

A. Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed using the procedure described by Laemmli (1970) using slab gel apparatus manufactured by Atto Co., Japan. A stock solution of 30% acrylamide containing 0.8% bisacrylamide, 1.0M Tris (pH 6 8 and 8.8) was prepared and mixed in the appropriate order and proportions to give a desired percentage of acrylamide It was then poured into the mold formed by two glass plates (7x9 cm) separated by 1.5mm thick spacer. Bubbles and leaks were avoided A comb providing templete for 12 sample wells was inserted into the stacking gel solution before polymerization began. When polymerization was completed in about 2 h, the comb was removed and wells were overlaid with distilled water. Proteins samples were prepared to give final concentration of 0 0625M Tris HCl pH 6.8 and 10% (v/v) glycerol and traces of bromophenol blue as a tracking dye Electrophoresis was performed in electrode buffer containing 0.025M Tris, 0 2M glycine at 100 Volts till the tracking dye reached the bottom of the gel

B. SDS Polyacrylamide Gel Electrophoresis

Tris glycine system of Laemmli (1970) was followed using a mini slab gel apparatus manufactured by Atto Co., Japan Stock solutions of 30% acrylamide containing 0.8% bisacrylamide, 1 5M Tris (pH 6.8 and 8.8) and 10% of SDS were prepared and mixed in the specific proportion to give desired percentage of acrylamide. The cocktail was then poured into the mold formed by two glass plates (7x9 cm) separated by 1.5 mm thick spacer. Bubbles and leaks were avoided A comb providing template for 12 sample wells was inserted into the stacking gel solution before polymerization began. The polymerization was complete in about 2 h The comb was removed and the wells thus formed were overlaid with distilled water. Protein samples were prepared to give final concentration of 1% (w/v) SDS 0.5% (v/v) ß-mercaptoethanol, 0.25M Tris HCl, pH 6.8 and 10% (v/v) glycerol and a trace of bromophenol blue as a tracking dye. Samples were than heated in a boiling water bath for about 3-5 min. 10 µl of protein samples were applied to the wells Electrophoresis was carried out at 100 volts for approximately 3 h in Tris Glycine buffer containing 0.025M Tris, 0.2M glycine and 0.2% SDS (pH 8.3). The protein bands were detected by staining with coomassie brilliant blue R-250.

C. Staining Procedures

(i) Coomassie brilliant blue staining

After completion of electrophoresis, the gels were stained for protein with coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 4 h. The gel was destained for several hours with 10% glacial acetic acid until the blue back ground became clear

(ii) Staining for HRP activity

When the electrophoresis was completed, the gel was transferred to the staining solution which is consist of 10 ml of 5 7mM O-diansidine-HCl and 21mM hydrogen peroxide in distilled water After 3-5 min incubation, it was thoroughly washed with distilled water and finally stored in distilled water

V. COLORIMETRIC ANALYSIS

A. Protein Estimation

The procedure described by Lowry *et al.* (1951) was followed A suitable aliquot of the protein sample was diluted to 1 0 ml with distilled water. To this 5.0 ml of freshly prepared copper reagent was added. The copper reagent prepared by mixing 0 5% copper sulphate in 1% (w/v) soidum potassium tartarate and 2% (w/v) sodium carbonate in 0.1N NaOH, in 1:50 ratio. After incubation for 10 min at room temperature, 0.5 ml of 1N Folin's reagent was added The contents were rapidly mixed and color intensity read after 30 min against a reagent blank at 660 nm. A standard curve prepared using BSA was used to calculate the concentration of protein

B. Total Carbohydrate Estimation

The procedure described by Dubois (1956) was followed Two millilitres of sugar solution containing between 10 to 70 μg of carbohydrate was pipetted into a test tube and 0 05 ml of 80% phenol added This was followed by the addition of 5 0 ml of concentrated sulphuric acid, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing

The tubes were allowed to stand for 10 min then they were shaken and again incubated for 20 min. at 30°C. The color intensity was measured at 490 nm. For the quantitation of hexose content glucose was used as the standard.

C. Determination of Amino groups

The procedure was essentially that described by Moore and Stein (1948). Appropriate aliquots of protein samples (0.1 ml) were placed in test tubes and 1.0 ml ninhydrin solution was added. (The ninhydrin solution was prepared by addition of 0.8 gm $SnCl_2.2H_2O$, in 500 ml of 0.2M sodium citrate buffer, pH 5.0. This solution was added to 20 0 gm of ninhydrin dissolved in 500 ml of methyl cellosolve) The tubes were heated for 20 min. in a boiling water bath. This was followed by the addition of 5.0 ml of diluent to each tube and content was mixed properly. The diluent was prepared by mixing equal volumes of water and η-propanol. Absorbance was taken in a spectrophotometer at 570 nm. Appropriate corrections for blank were made either by reading blank solution against diluent and zeroing the instrument on the blank reading so obtained or by mean of an appropriate blank solution in conventional manner. A standard curve prepared using leucine was used for quantitation of amino groups.

VI ENZYME ASSAYS

A. Peroxidase

Peroxidase was assayed at 37°C by measuring the initial rate of oxidation of O-diansidine HCl by hydrogen peroxide using the two substrates in saturating concentrations (Ugarava *et al.*, 1979) To, 2 3 ml of 0.1M sodium phosphate buffer, pH 7.0 was added 0 07 ml 5 7mM

O-diansidine-HCl in distilled water, 0.07 ml 21mM hydrogen peroxide and 0.05 ml of the 0.1-1.5μM enzyme solution The reaction was stopped by addition of 1 0 ml of 6 0N HCl Absorbance was recorded in a Beckman - Spectrophotometer at 460nm (ϵ460 = 30,000 M^{-1} cm^{-1}) The quantity of O-diansidine HCl (μmoles) oxidized per min by the enzyme was assumed to be measures of the enzyme activity The enzyme activity of the peroxidase at elevated temperature was assayed as shown above.

One unit of enzyme activity is the amount that catalyses the oxidation of 1 μmole of O-diansidine-HCl per min. at 37°C.

B. Invertase

Invertase activity was estimated using procedure described by Bernfeld (1955). The reaction mixture contained in a total volume of 0.3 ml, 150 μl of 0.2M sodium acetate buffer, pH 4 8, 100 μl of suitably diluted enzyme solution and 50 μl of 0.5M sucrose solution The assays mixture was incubated at 37°C for 10 min and the reaction stopped by the addition of 0.2 ml of 0.5M sodium phosphate buffer, pH 7 0 followed by incubation at 100°C for 3 min One ml dinitrosalicylic acid reagent was added to the reaction mixture and the tubes were incubated at room temperature for 5 min. After heating in boiling water bath for 5 min., the tubes were cooled and 3.0 ml distilled water was added to the reaction mixture. The intensity of colour was read at 540 nm

One unit of invertase is the amount that hydrolyzes 1 0 μmoles of sucrose to glucose and fructose per 10 min at 37°C

C. Glucose Oxidase

Glucose oxidase was assayed by the procedure described by

Hatton and Regoeczi (1976) as modified by Iqbal and Saleemuddin (1983a). Glucose was prepared as 0.15M solution Peroxidase and O-diansidine-HCl were added to 0.05M sodium phosphate buffer, pH 6.1, to give a final cone of 0.01%. One millilitre of glucose solution was added to 1.8 ml of freshly prepared peroxidase/O-diansidine-HCl reagent After incubation at 37°C for 2 min., 0.2 ml of suitably diluted enzyme was added and the reaction was allowed to proceed for 15 min at 37°C The reaction was terminated by the addition of 1 0 ml of 4.5M sulphuric acid and the absorbance recorded at 550 nm.

One unit of glucose oxidase is the amount that oxidizes, 1.0 µmole of gluconic acid and hydrogen peroxide per 15 min at pH 6.1 at 37°C.

D. Amyloglucosidase

Amyloglucosidase activity was determined using the procedure described by Rao *et al.* (1981) with some minor modifications This procedure also involves the use of glucose oxidase in combination with peroxidase and O-diansidine-HCl to estimate glucose released during the enzymatic hydrolysis of starch or maltose The reaction mixture contained in a total volume of 1.0 ml, 400 µL of 0.1M sodium acetate buffer, pH 4.5, 100 µL enzyme in the same buffer and 0.5 ml of 0.1M maltose or 2% starch solution. The assay mixture was incubated at 37°C for 30 min. and reaction terminated by heating in boiling water bath for 3 min The tubes were brought subsequently to 37°C and glucose released was determined. 2.0 ml of solution C was added to the tubes. The solution C prepared by adding the solution A and B to 85 ml of 20% glycerol (solution A contained 10 mg glucose oxidase and 2 5 mg peroxidase in 10 ml of 0 1M sodium phosphate buffer, pH 7 0 and

solution B contained 30 mg O-diansidine-HCl in 5 0 ml distilled water) The reaction was terminated by the addition of 2.0 ml of 6 0N HCl and absorbance read at 540 nm

One unit of amyloglucosidase is the amount that releases 1 0 μmole of glucose from maltose or starch per 30 min at 37°C

E. Insoluble Enzymes

Insoluble HRP, invertase, Glucose oxidase and amyloglucosidase were assayed in a manner similar to the respective soluble preparations except that the assay mixture was continuously agitated during the incubation periods. Prior to transfer the insoluble enzyme preparation were made into suitably diluted suspension that could be reproducibily pipetted out.

Chapter III

INACTIVATION AND REACTIVATION OF VARIOUSLY IMMOBILIZED HRP

RESULTS

Preparation of immobilized HRP

It is now well established that nature of association of an enzyme with support has remarkable influence on the properties of the immobilized preparation. In order to obtain useful immobilized preparations of HRP, the strategies involving coupling of the enzyme to the Sepharose support were investigated. These included those in which HRP was linked both via its amino acid side chain amino groups and through its carbohydrate moieties to the Sepharose matrix. The immobilized preparations studied were (i) Sp-HRP in which HRP was coupled to Sepharose exclusively through the amino acid side chain amino groups, (ii) Sp-NHHRP that presumably contained HRP coupled both by amino acid side chain amino groups and through additional amino groups incorporated in the glycosyl chains, (iii) SpNH-COHRP in which HRP is expected to be linked exclusively via carbohydrate spikes that were treated with periodate to generate aldehydic groups, and (iv) SpCon A-HRP, a preparation containing native HRP affinity bound on Con A-Sepharose A diagramatic representation of these preparations has been indicated in Figure 3.1

Unmodified enzyme was used for the preparation of Sp-HRP and Sp Con A-HRP, Sepharose matrix was activated with cyanogen bromide for the coupling of enzyme in the former while it was precoupled with Con A for obtaining the later. The enzyme was treated with 10mM sodium (meta) periodate for the preparations of Sp-NHHRP and SpNH-COHRP The enzyme lost 30% carbohydrate and about 40% enzyme activity as a result of this treatment Amination of the periodate treated enzyme with ethylenediamine resulted in the incorporation of additional 17 moles of amino groups per mole of enzyme (Table 3 1)

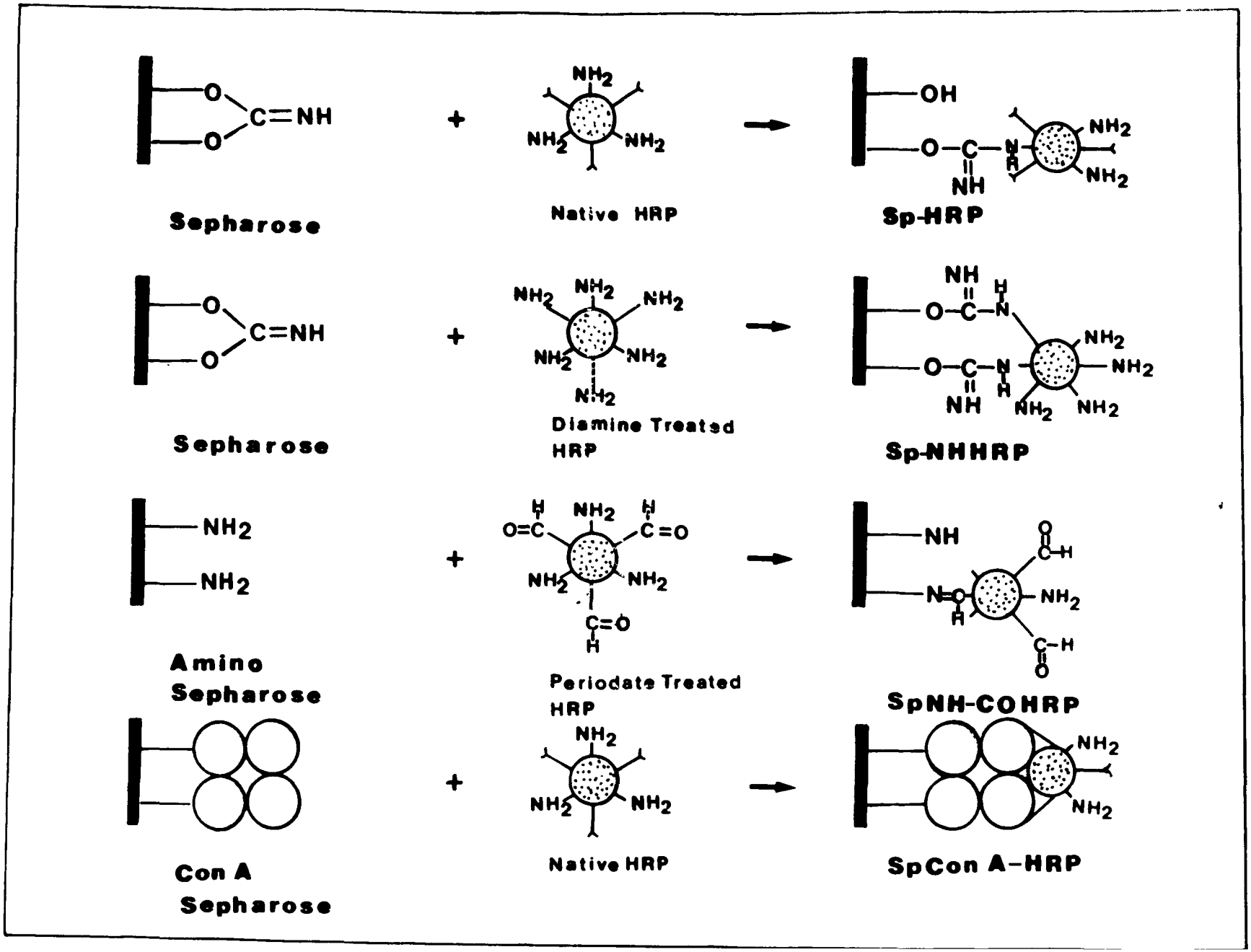

Sepharose
Native HRP
Sp-HRP
Sepharose
Diamine Treated HRP
Sp-NHHRP
Amino Sepharose
Periodate Treated HRP
SpNH-COHRP
Con A Sepharose
Native HRP
SpCon A-HRP

Figure 3.1

Schematic representation of the likely linkages between the enzyme and support as a result of immobilization on Sepharose using various procedures.

Table 3.1

Activity, carbohydrate and amino acid content of periodate and ethylenediamine treated HRP*

Commercial preparation of HRP was treated with 10mM sodium (meta) periodate followed by 100mM ethylenediamine as described in the methods. Enzyme acitivity, protein, carbohydrate and amino group content of the native and modified enzyme was determined.

Preparations	Specific Activity U/mg Protein	carbohydrate µmoles/mg Protein	Amino groups moles/mole of Protein
Native	50083	2.1	28.6
Periodate treated	28011 (56)	1.5 (71)	45.0

* Each value represent the mean of atleast four independent experiments performed in duplicate

Values in perenthesis represent percent of those of the native enzymes.

Table 3.2 describes the quantities of HRP associated with Sepharose and 'η' values of various immobilized preparations Highest yield of immobilization was obtained in the preparation Sp-NHHRP that contained HRP bound via both of its amino acid side chain amino groups as well as through amino groups incorporated into the carbohydrate moieties The preparation also exhibited the highest 'η' value Contrary to earlier observations made with several other glycoenzymes, the Con A-Sepharose bound relatively small amount of HRP Preparation SpNH-COHRP also indicated low yield of immobilization. Taking into consideration the risk of elution of the non covalently associated HRP from Con A-Sepharose at high temperature and other denaturing conditions together with its low activity, the SpCon A-HRP preparation was not included in inactivation and reactivation studies.

Inactivation and reactivation

Native HRP is rather stable to heat inactivation. Immobilization however, conferred additional stability and the immobilized preparation necessitated rather high temperature to achieve significant inactivation As shown in Figure 3.2, the immobilized preparations were far more stable than the soluble HRP. The soluble enzyme lost essentially complete activity in 2 h at 70°C, while Sp-HRP, Sp-NHHRP and SpNH-COHRP retained 15, 50 and 10% activity respectively under identical conditions As evident from the Figure 3.2, the heat treated Sp-NHHRP preparation regained almost complete activity in about 6 h when incubated at 0°C The regain in activity in case of preparations Sp-HRP and SpNH-COHRP was far lower The native enzyme did not regain significant activity under these conditions

As the preparation Sp-NHHRP lost relatively small fraction of

Table 3.2

Immobilization yield of HRP by various procedures.[a]

Sepharose support	Enzyme	units bound/g[d]		'η' value
		Theoretical (A)	Actual (B)	(B/A)
CNBr-Activated[b]	Native	1500	900	0.60
CNBr-Activated	Diamine adducted	2000	1850	0.92
CNBr-Activated+Diamine treated	Periodate treated	1150	600	0.52
CNBr-Activated+Con A treated[c]	Native	900	800	0.88

a - Each value represents the mean of atleast four independent experiments performed in duplicate.

b - Each gram of Sepharose was treated with 200 mg cyanogen bromide for 10 min. at 4°C.

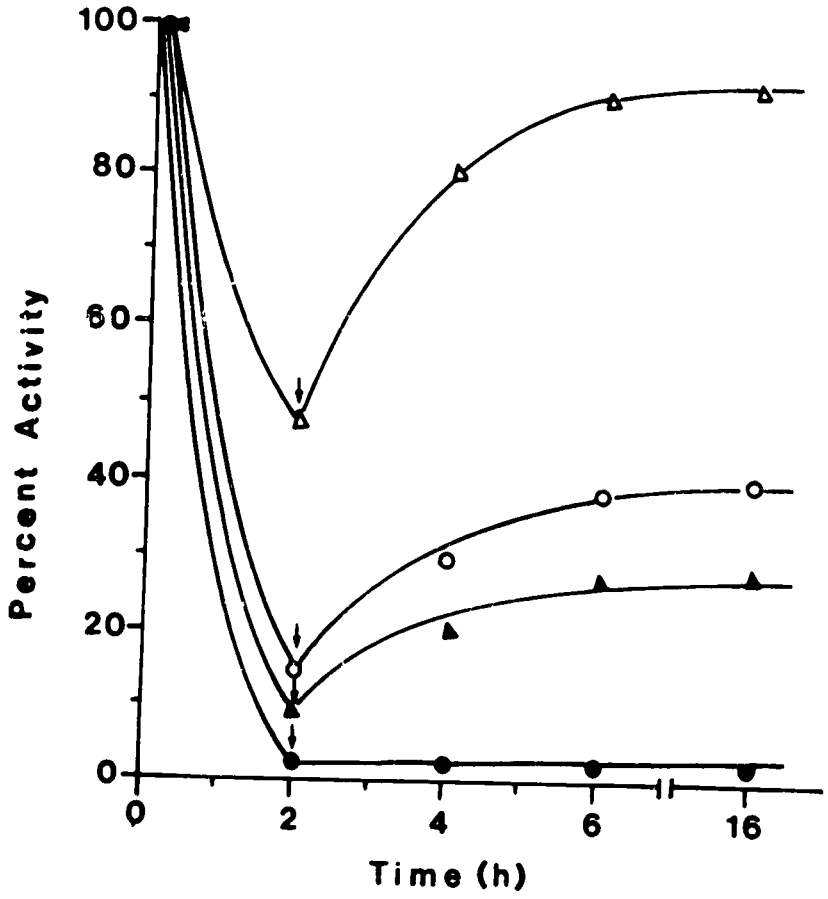
c - 10 mg Con A was coupled to one gram of Sepharose.

d - About 2500 units of HRP were added to each gram of matrix and units unbound and those in washing were substracted from the added units to obtain "Theoretical" binding, an appropriate aliquot of the immobilized preparation was assayed to obtain the "Actual" activity.

Figure 3.2

Effect of temperature on inactivation and reactivation of soluble and immobilized HRP preparations.

Approximately 200 units of soluble or immobilized preparation were incubated at 70°C in 0.01M sodium phosphate buffer, pH 7.0, for 2 h. Aliquots of each preparation were removed and enzyme activity determined under standard conditions. These preparations were subsequently shifted to 0°C for reactivation and HRP activity determined at various time intervals. Arrow indicates the time point at which the preparation was transferred to 0°C for further incubation. (●), soluble HRP; (o), Sp-HRP (▵), Sp-NHHRP; (▲), SpNH-COHRP.


100
80
60
40
20
0
Percent Activity
0
2
4
6
16
Time (h)

activity even at 70°C, attempts were made to achieve higher degree of inactivation by incubating the preparation at higher temperature with a view to further investigate their ability to regain activity after nearly complete inactivation The soluble and immobilized preparations were exposed to 75°C for 30 min. (Figure 3.3). As a result of this treatment soluble HRP was inactivated completely while the immobilized preparations retained significant enzyme activity The Sp-NHHRP was most stable and retained 29% activity The preparation also recovered highest activity on subsequent incubation at 0°C, with a maximum recovery of 60% of initial enzyme activity. The regain in activity in case of preparations Sp-HRP and SpNH-COHRP were only 40 and 20% respectively (Figure 3.3) Further incubation upto 16 h at 0°C resulted in no significant increase in the activity. The extent of reactivation achieved at 75°C was however, far lower as compared to that achieved at 70°C. This appears to be a consequence of irreversible alteration of the enzyme at 75°C. Experiment in which these preparations were inactivated for a longer duration at 75°C further support this observation Incubation of both soluble and immobilized HRP preparations at 75°C produced almost complete inactivation in 2 h. Immobilized preparations treated thus regained a very little enzyme activity even after 16 h incubation at 0°C. Maximum reactivation, however, was observed in Sp-NHHRP, that regained about 20% of initial enzyme activity (Figure 3.4)

The ability of the immobilized HRP preparations to recover enzyme activity was inversely related to the length of the heat treatment As shown in Figure 3.5, both Sp-NHHRP and Sp-HRP regained progressively lower percent activity as the length of exposure to 70°C increased The Sp-NHHRP retained relatively greater fraction of initial activity after heat treatment and also recovered more catalytic activity after subsequent

Figure 3.3

Effect of exposure to 75°C on inactivation and subsequent reactivation of soluble and immobilized HRP.

Approximately 200 units of soluble or immobilized HRP preparation were incubated in 0.01M sodium phosphate buffer, pH 7 0 in a total volume of 1.5 ml for 30 min. The preparations were quickly transferred to ice and further incubated for various durations. After incubation in ice the prepartions were brought to 37°C and HRP activity assayed by standard procedure as described in the text. Arrows indicate the time point at which the samples were transferred to ice. (●), Soluble HRP; (o), Sp-HRP; (▵), Sp-NHHRP; (▲), SpNH-COHRP.

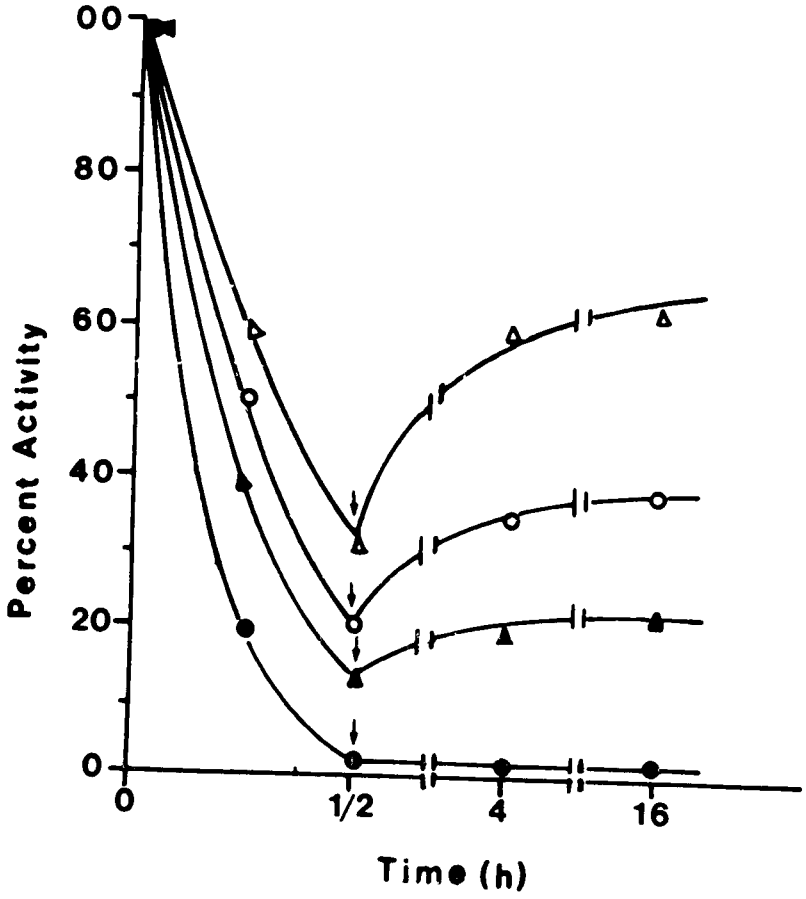

00
80
60
40
20
0
Percent Activity
0
1/2
4
16
Time (h)

Figure 3.4

Inactivation and reactivation of soluble and immobilized preparations at 75°C.

Soluble or immobilized preparation was incubated at 75°C for 2 h Aliquots were removed to determined the activity The preparations were shifted to 0°C for reactivation (●), soluble HRP, (o), Sp-HRP, (▵), Sp-NHHRP; (▲), SpNH-COHRP

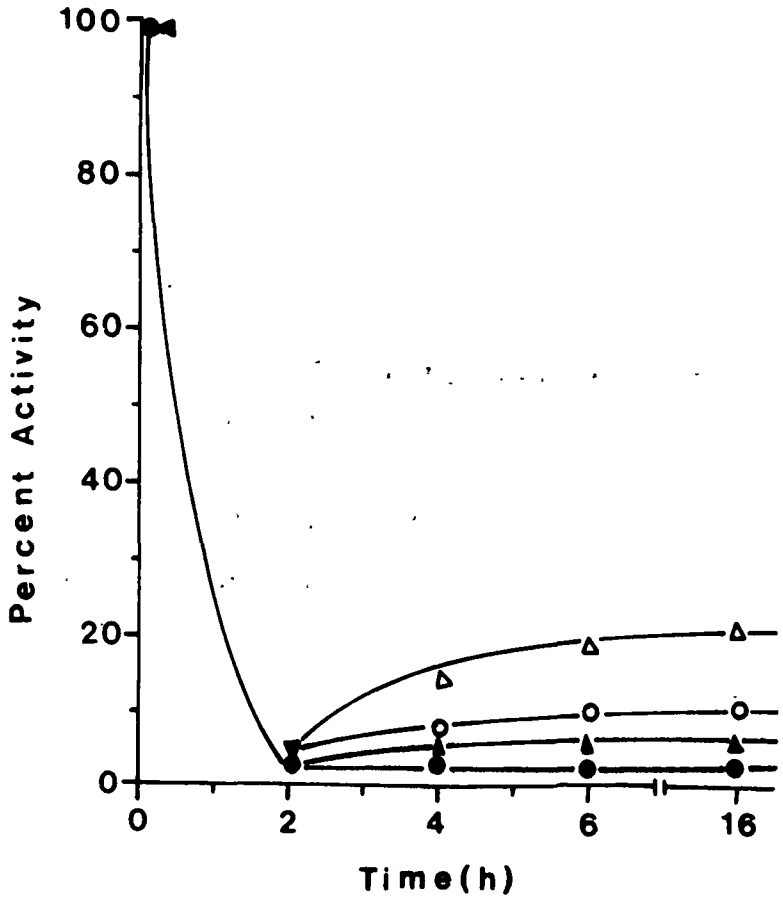
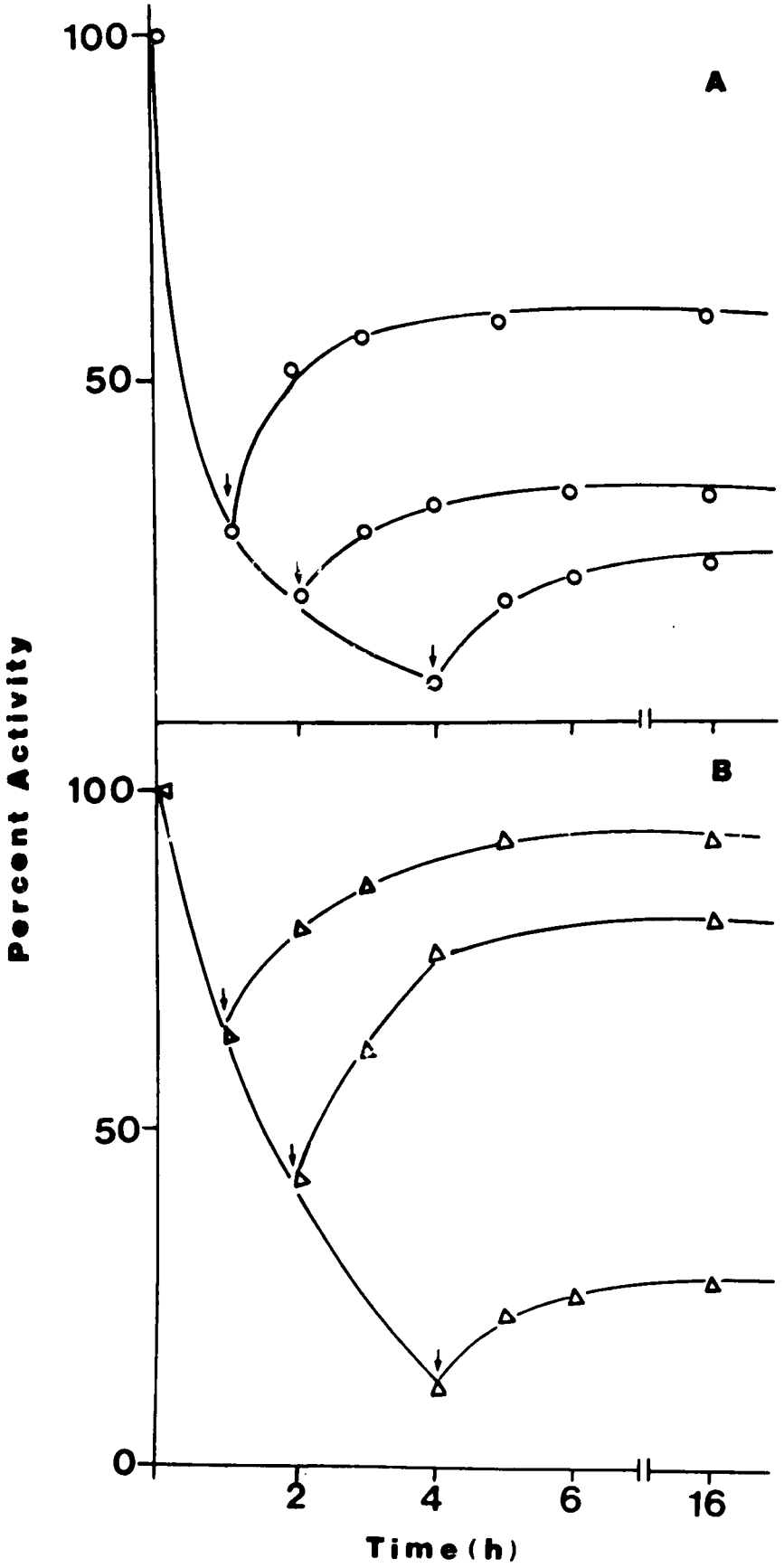

100
80
60
40
20
0
Percent Activity
0
2
4
6
16
Time(h)

Figure 3.5

Effect of length of exposure at 70°C on the inactivation and reactivation of Sp-HRP (A) and Sp-NHHRP (B).

Immobilized preparations Sp-HRP and Sp-NHHRP (approximately 200 units) were incubated at 70°C for various durations The preparations were subsequently transferred to 0°C for reactivation Arrows indicate the time points at which the samples were transferred to 0°C Various HRP preparations are indicated by symbols as detailed in the legend to Figure 3.2


100
50
A
100
B
50
0
Percent Activity
2
4
6
16
Time (h)

incubation at 0°C.

Calcium ions have been shown to stabilize many enzymes against heat and other forms of denaturation. Attempts were therefore made to study the role of calcium ions on the thermal inactivation and reactivation of HRP As shown in Figure 3.6, when 10mM calcium was included in the preparation during the heat treatment, thermal stability of the native HRP at 75°C was only marginally increased. The immobilized preparations, however, showed remarkable increase in their stability in presence of calcium. After 3 h incubation at 75°C, the preparation Sp-HRP and Sp-NHHRP and SpNH-COHRP retained about 30, 50 and 20% activity respectively. However, in the absence of calcium these preparations lost about 95, 90 and 97% activity respectively.

Effect of urea

In contrast to the observations made with heat inactivation, Sp-HRP recovered greater fraction of initial enzyme activity after urea denaturation (Figure 3.7). 8.0M urea caused complete inactivation of native and immobilized preparations the complete removal of urea by washing with urea free buffer did not result in any significant recovery of activity. Incubation of urea denatured preparation with thiol reductant followed by the removal of denaturant and subsequent incubation with oxidized and reduced glutathione mixture was also attempted in order to achieve the reactivation of the preparation. Such attempts however, resulted only in marginal or moderate recovery of enzyme activity. Very little regain in activity was observed in calcium free media and it was essential to include calcium ions in the reactivation media. As evident from Figure 3.7, the Sp-HRP, regained about 65% activity while the Sp-NHHRP recovered only about 25% activity in about 4 h at 0°C in the calcium containing reactivation media.

Figure 3.6

Effect of calcium on inactivation of soluble and immobilized HRP preparations.

Soluble or immobillized preparation (Approximately 200 units) in 0.01M Tris HCl buffer, pH 7.0, was incubated at 75°C for various durations. Aliquots were removed to determine the activity as described in text. (o ●), soluble HRP; (Δ▲), Sp-HRP; (□■), Sp-NHHRP; (◇◆), SpNH-COHRP. Open symbols represent preparations incubated in absence of calcium while filled symbols represent those incubated with calcium

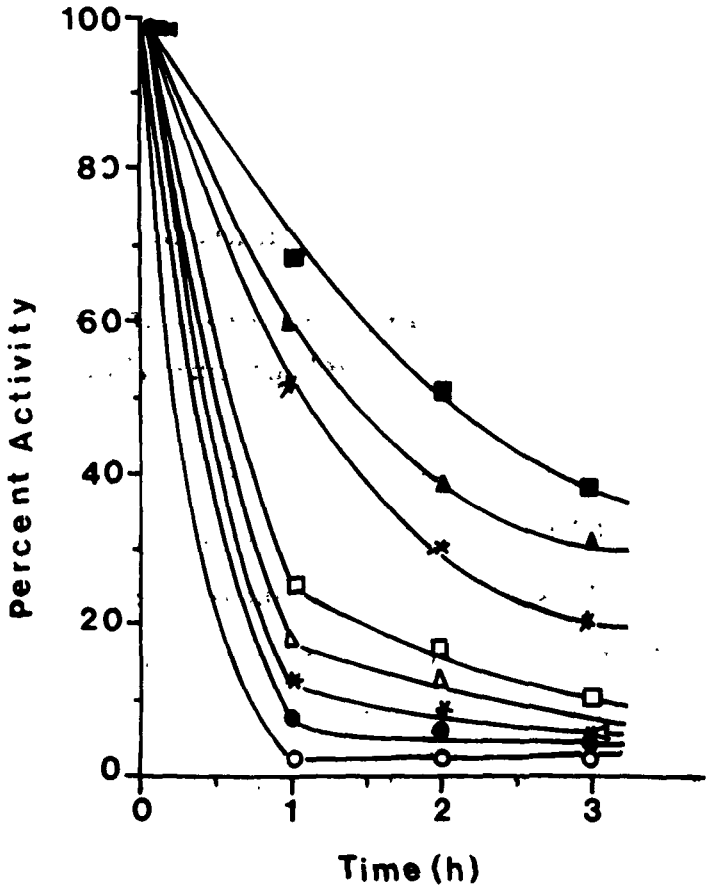
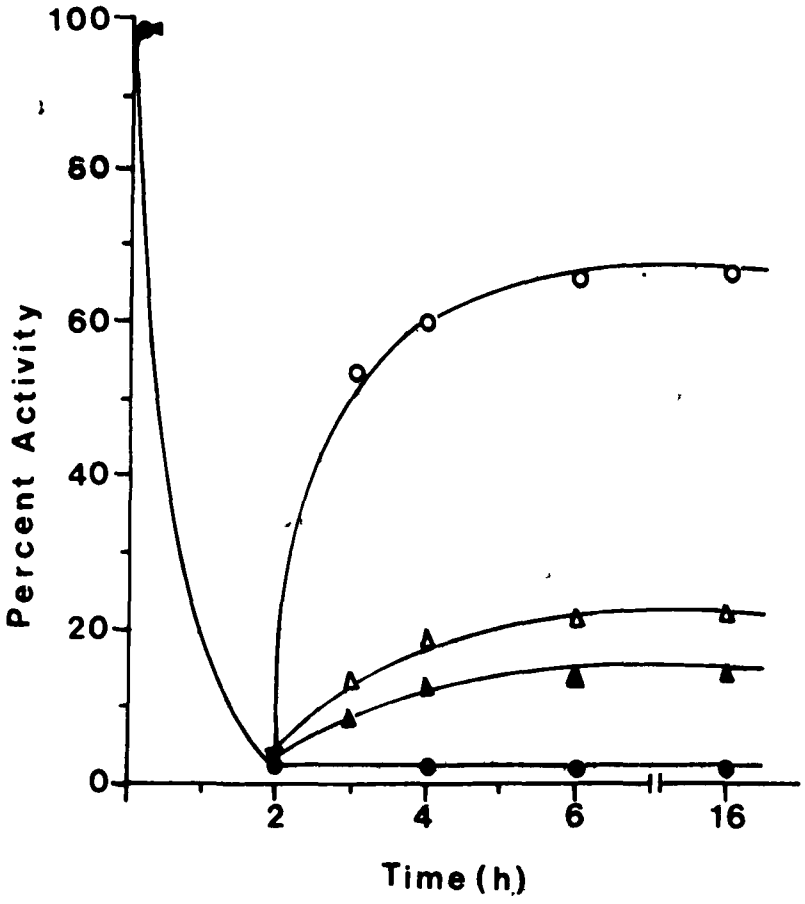

100
80
60
40
20
0
Percent Activity
0
1
2
3
Time (h)

Figure 3.7

Reactivation of native and immobilized HRP preparations treated with urea and dithiothretol.

Approximately 250 units of native or immobilized HRP preparaions in 0.01M Tris HCl buffer, pH 7.0 were made 8.0M with respect to urea, 20.0mM with dithiothretol and 5.0mM with EDTA in a total volume of 5.0 ml. The tubes were incubated at 4°C for 2 h. Urea and dithiothretol were rapidly removed from the immobilized preparations by centrifugation and repeated washings with the buffer. The preparations were finally placed in 0.01M Tris HCl buffer, pH 7.0 containing 2.0mM calcium at 4°C for various durations as indicated. Native HRP was dialysed against 0.01M Tris HCl buffer, pH 7.0 containing 2.0mM calcium for 16 h at 4°C. Alternatively all the samples were diluted 10-fold with buffer to decrease urea concentration and incubated at 4°C for the indicated intervals. There was very little difference between the activities regained in the preparations subjected to either treatment. Aliquots were removed to determine the activity at various time intervals. (●), soluble HRP; (o), Sp-HRP; (△), Sp-NHHRP; (▲), SpNH-COHRP.


100
80
60
40
20
0
Percent Activity
2
4
6
16
Time (h)

DISCUSSION

HRP is one of the most widely used enzyme in view of its convenient assay procedure, ready availability and high stability. It is extensively used in enzyme linked immuno sorbent assays (ELISA) and in the visualization of various polypeptides after blotting from polyacrylamide gels using specific antibodies, lectins etc. in ELISA and for microdetection of the enzyme on nitrocellulose, peroxidase is used as a conjugate, albeit soluble, with other proteins and hence can be considered immobilized HRP is a hemoprotein in which heme moiety is covalently associated with protein and some of isoenzyme forms also require calcium for catalytic activity. Attempts were made therefore, to investigate the effect of the immobilization and calcium on the stability of HRP and ability to recover enzyme activity after inactivation of the peroxidase preparations.

Immobilization procedure involving covalent coupling of the enzymes to solid support however, are not useful in case of glycoenzyme that have the side chain amino groups masked by carbohydrate spikes (Hsiao and Royer, 1979). Attention was therefore, directed towards strategies in which carbohydrate spikes form the point of attachment with support. Several investigators obtained useful glycoenzyme preparations, immobilized via the glycosyl residues (Zaborsky and Ogletree, 1974; Woodward and Zachry, 1982; Hsiao and Royer, 1979).

Periodate treatment under the conditions used for obtaining preparations Sp-NHHRP and SpNH-COHRP, resulted in about 40% loss of catalytic activity and about 30% loss of carbohydrates (Table 3 1) In view of the high concentration of ethylenediamine used for amination of periodate treated enzyme possibility of the diamine reacting with both of its amino groups with the aldehydic groups of the enzyme is expected to be low

While the involvement of glycosyl residues in the catalytic function of HRP have not been established, they seem to be somehow involved in the catalytic function of the peanut peroxidase (Hu and Van Huystee, 1989) Although peroxidation of other enzymes has also been shown to cause varying degree of inactivation, loss of activity could be minimized by carrying out the reaction under mild conditions (Marek *et al.*, 1984)

Among the HRP preparations investigated highest yield of immobilization as well as highest 'η' value were obtained with Sp-NHHRP (Table 3.2). The high immobilization yield may be attributed to availability of larger number of exposed amino groups for coupling to the support Since oligosaccharidesof HRP are localized on the surface of the enzyme (Welinder, 1985), the amino groups incorporated into these would be more accessible for reaction with cyanogen bromide activated Sepharose than those of the amino acid side chain amino groups. The amino groups are also expected to be far removed from the active site of the enzyme in view of the location on the glycosyl chains. This contention is supported by the observation that Sp-NHHRP preparation exhibits very high 'η' value, an indication of the very high accessibility of the active site of the enzyme for the substrate (Table 3.2). When the amino groups substituted matrix was reacted with the periodate treated enzyme, however, very low level of immobilization was achieved (Table 3.2). This may be related to the formation of the covalent adducts of the periodate treated enzyme molecule involving amino groups of one enzyme molecule with the generated aldehydic groups of another. Such a reaction will decrease the aldehydic groups and interfere with the coupling of enzyme to solid support Unlike several other glycoenzyme however, the yield of immobilization of Con A support was very low This may be related to poor affinity of some HRP forms for Con A (Derbyshire, 1973)

In support of the earlier observations (Hsiao and Royer, 1979; Woodward and Zachry, 1982; Saleemuddin & Husain 1991), the Sp-NHHRP preparation was far more thermostable than the Sp-HRP preparation, Sp-HRP inturn was far more stable than the soluble HRP. Due to the risk of desorption of HRP bound to Con A-Sepharose at high temperatures, the Sp Con A-HRP preparation was not used in these studies. The Sp-NHHRP not only retained maximum activity after heat treatment but also recovered upto 60% of initial activity on incubation at 0°C (Figure 3.3). Incubation with mixturesof reduced and oxidized glutathione that facilitate recovery of enzyme activity after inactivation did not help in increasing the reactivation of heat inactivated HRP.

Exposure to a higher temperature (75°C) for 2 h resulted complete inactivation of all preparations. The recovery in activity after incubation at 0°C was however, poor in all the preparations suggesting irreversible inactivation (Figure 3.4). The ability of the HRP preparations to recover enzyme activity was inversely related to the length of the heat treatment. As shown in Figure 3.5, both Sp-HRP and Sp-NHHRP preparations regained less and less of the initial enzyme activity as the length of exposure to 70°C increased. At all the time points investigated however, Sp-NHHRP retained greater activity and regained more activity after subsequent incubation at 0°C. A large number of glycoenzymes have been shown to exhibit markedly by higher stability if they are immobilized via glycosyl residues rather than amino acid side chain amino groups (Hsiao and Royer, 1979; Iqbal and Saleemuddin, 1983b). Lack of complete recovery of enzyme activity after denaturation of HRP may be result of a covalent modification such as hydrolysis of asparagine or entrapment of the enzyme molecules in a stable non native conformations (Klibanov and Mozhaev, 1978).

In view of the observations that HRP and other plant peroxidases contain bound calcium (Haschke and Friedhoff, 1978, Aibara *et al.*, 1982), the effect of calcium ions on the stability against urea denaturation and ability to recover enzyme activity after 8.0M urea treatment was investigated. As evident from Figure 3.6 inclusion of 10mM calcium ions increase the stability of the native and more significantly of the immobilized preparations. Comparable stabilization was also observed when the calcium concentration was lowered to 2mM Best stabilization was observed in case of Sp-NHHRP. Haschke and Friedhoff (1978) demonstrated that calcium ion free HRP is far more labile as compared to that containing bound calcium. Stabilizing effects of calcium ion as a number of other enzymes against various forms of denaturation has been reported (Gomez *et al.*, 1977; Delaage and Lazdunski, 1967; 1968; Martin and Frazier, 1963). In case of trypsin calcium ion mediated effects were shown to be due to stabilizing effects on the tertiary structures and ability of the cation to prevent autolysis (Gomez *et al.*, 1977). The greater protection observed in case of immobilized preparations and maximum stabilization observed in case of Sp-NHHRP suggest that stability against heat inactivation and protective effect of calcium complement each other

Evidently, the denatured enzyme loses calcium readily (Haschke and Friedhoff, 1978) and fails to refold unless adequate amount of these ions are provided in the sample. A large number of other enzyme required calcium for refolding to the native state after denaturation induced by various agents (Rao and Brew, 1989)

In contrast to observation made with heat inactivation, Sp-HRP but not Sp-NHHRP recovered greater fraction of initial enzyme activity after urea denaturation (Figure 3.7). It was essential to include calcium in the incubation mixture as very little activity was regained in its absence. It is

difficult to explain the superiority of Sp-NHHRP in recovering greater activity after heat inactivation and of Sp-HRP after urea inactivation Evidently, it may be related to the difference in nature of linkage with the support in some inexplicable way is responsible for the behaviour Unfolding achieved by heat treatment primarily disrupts hydrogen bonds and electrostatic interaction and may actually stabilize hydrophobic forces while urea treatment is known to cleaves all non covalent linkages. Studies of Monsan and Combes (1988) and Iqbal and Saleemuddin (1983b) have shown that stability of the immobilized preparations is greatly influenced by the number of linkages between enzyme and support It is quite likely that Sp-NHHRP may be linked at more sites with the support due to the presence of larger number of accessible amino groups. Such molecules of HRP may be rigid and resist denaturation However, it has been demonstrated by several workers that reactivation of heat denatured enzymes to their native form is facilitated if the polypeptide is first completely unfolded prior to exposing it to conditions that facilitate refolding (Klibanov, 1979). The relative low reactivation yield observed subsequent to urea denaturation in case of Sp-NHHRP may be related to its inability to unfold completely in urea due to attachment at several sites on the support and consequently to regain the native conformation.

Chapter IV

STUDIES ON THE BEHAVIOUR OF ANIONIC AND CATIONIC HRP IMMOBILIZED BY VARIOUS PROCEDURES

RESULTS

Isolation and purification of HRP isoenzymes

Commercial HRP is a mixture of several anionic and cationic isoenzymes. These were purified by ion exchange chromatography to yield one cationic and one anionic form HRP isolation and purification procedure used in this study was based on that described by shannon *et al.* (1966). The anionic isoenzymes of HRP do not bind to CM-cellulose and emerge in the flow through, while cationic HRP forms were bound and retained. The bound fractions were then eluted with a linear gradient of 5-250mM sodium acetate buffer, pH 4 4 As shown in Figure 4 1, three cationic isoenzymes were obtained and designated as B, C, and D in the same order These were further subjected to rechromatography on a CM-cellulose column until the elution pattern indicated clear separation of individual fraction. Isoenzymic form C that eluted as the major peak was used for further study. Purity of this form was ascertained by electrophoresis (data not shown). The individual anionic isoenzymes were isolated by chromatography on DEAE-cellulose column As shown in Figure 4.2, two peaks designated as A-1 and A-2 were obtained, when the DEAE column was eluted by a linear gradient of O-100mM NaCl in Tris HCl buffer, pH 8.4. The fractions were also further purified by repeated chromatography to achieve clear resolution of the two forms Fraction A-2 representing the major anionic form was used in this study Purity of this isoenzymic form was further ascertained by PAGE and it migrated as single protein band (Figure 4 3)

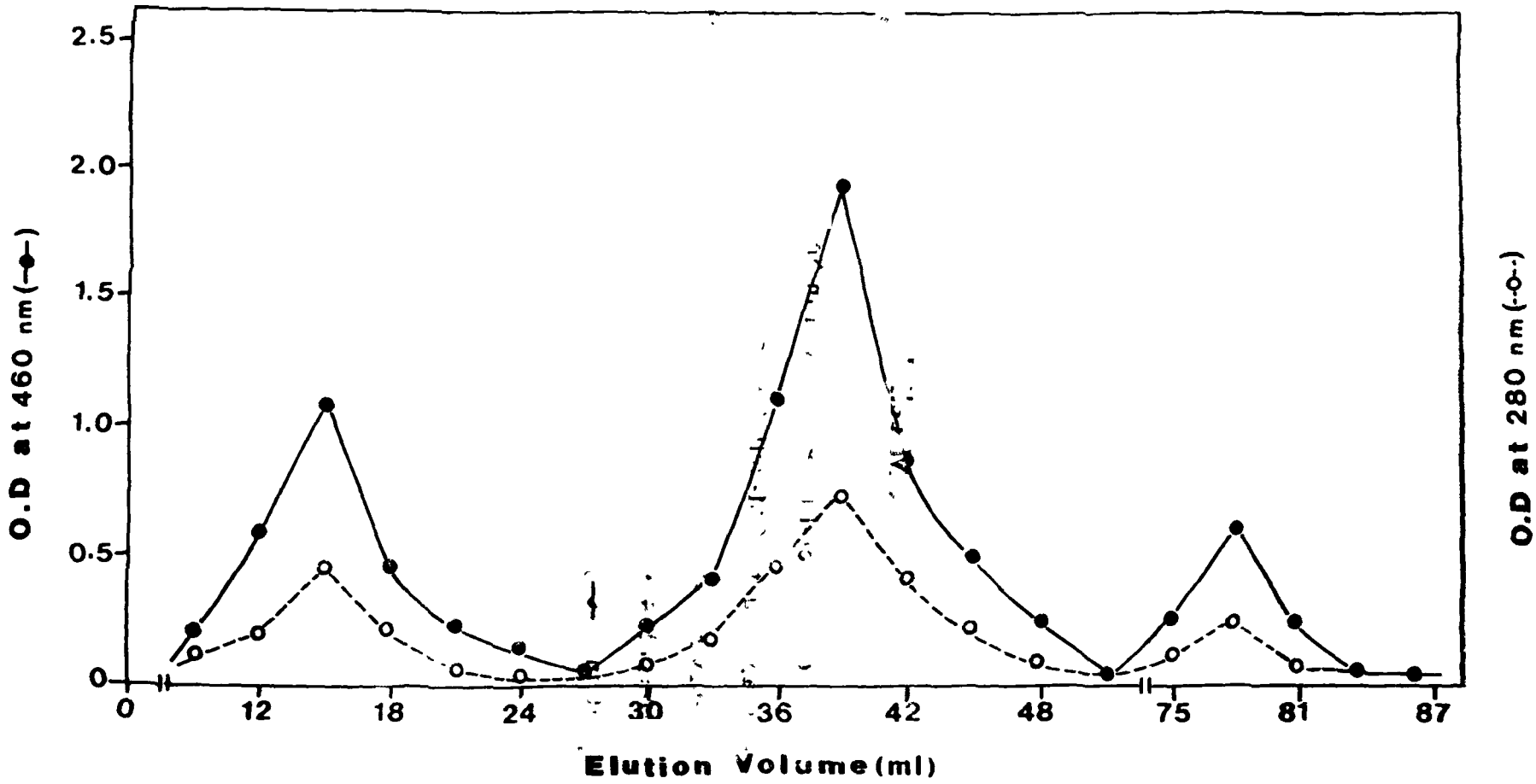
Immobilization of the anionic and cationic HRP isoenzymes

The anionic and cationic isoenzymic form of HRP were immobilized

Figure 4.1

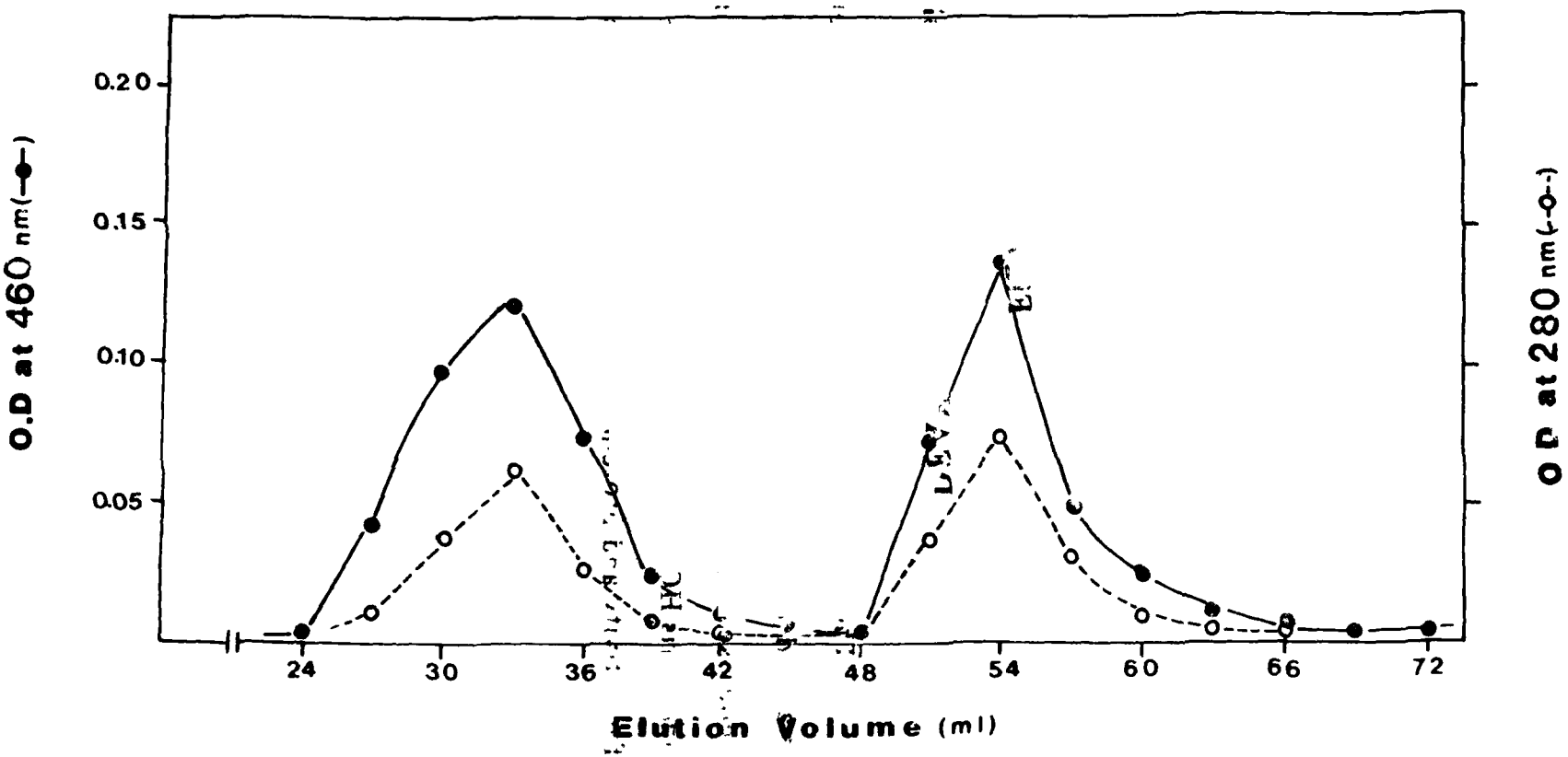
CM-cellulose chromatography

Commercial HPI dissolved in 5mM sodium acetate buffer, pH 4.4 was applied to CM-cellulose column and thorougly washed. Cationic isoenzymes were eluted with linear gradient of 5-250mM sodium acetate buffer, pH 4.4. 3.0 ml fractions were collected and assayed for activity and protein (-•-), HRP activity; (--o--), Protein.


2.5
2.0
1.5
1.0
0.5
0
O.D at 460 nm(—●—)
O.D at 280 nm(--o--)
0
12
18
24
30
36
42
48
75
81
87
Elution Volume(ml)

DEAE-cellulose chromatography

The fraction that was not retained on CM-cellulose column was dialyzed against 5mM Tris-HCl buffer, pH 8.4 and applied on DEAE-cellulose column. Anionic isoenzymes were eluted with 0-100mM NaCl in 5mM Tris-HCl buffer, pH 8.4. 3.0 ml fractions were collected and assayed for activity and protein. (—▲—), Esterase activity; (--o--) Protein


O.D at 460 nm(—●—)
O D at 280 nm(-o-)
0.20
0.15
0.10
0.05
24
30
36
42
48
54
60
66
72
Elution Volume (ml)


A
B
C
D

with via glycosyl residues as well as by amino acid side chain amino groups. Catalytic activity of both isoenzymes was significantly lowered as a result of treatment with 2mM sodium (meta) periodate (Table 4.1). The anionic HRP however, appeared to be somewhat more sensitive to periodate oxidation, with the treatment resulting in a loss of about 38% catalytic activity and 48% of carbohydrate. The cationic HRP isoenzyme lost about 24% catalytic activity and 36% carbohydrate under the conditions.

Amination of the periodate treated preparation with ethylenediamine resulted in the incorporation of additional 12 and 17 moles of amino groups per mole of anionic and cationic HRP respectively (Table 4.1). As discussed in the previous section the preparation Sp-HRP in which HRP was coupled to Sepharose exclusively through the amino acid side chain amino groups, preparation Sp-NHHRP, contained HRP coupled to Sepharose presumably both by amino acid side chain amino groups as well as through amino groups incorporated in the carbohydrate moieties. The preparation SpCon A-HRP, was coupled through carbohydrate residues of enzymes with Con A via affinity binding. While preparation SpCon A-COHRP, in which HRP expected to be coupled through generated aldehydic groups of HRP with amino acid side chain amino groups of Con A, precoupled to Sepharose.

Table 4.2 presents the amount of anionic and cationic HRP immobilized and 'η' values of the bound enzyme when the different strategies of immobilization were followed as discussed above. In both cases highest yield of immobilization was observed in the preparation Sp-NHHRP. This preparation also exhibited highest 'η' value for both isoenzymes.

Table 4.1

Activity, carbohydrate and amino group content of periodate and ethylenediamine treated HRP isoenzymes.

Anionic and cationic isoenzymes of HRP were treated with 2mM sodium (meta) periodate for 30 min. at 4°C in dark followed by 100mM ethylenediamine treatment as discussed in methods Enzyme activity, carbohydrate and amino groups of native and modified enzyme were determined

Preparations	Specific Activity (U/mg protein)	Cabohydrate µmoles/mg protein	Amino Groups moles/mole protein
Anionic			
Native	15202 ± 2.1	10.5 ± 1 6	32 ± 1.3
Periodate treated	9407 ± 1.8 (62)	5 5 ± 1 5 (52)	44 ± 1.0
Cationic			
Native	48146 ± 1.7	9 ± 1.5	35 ± 1.1
periodate treated	36580 ± 1.5 (76)	5.8 ± 1 2 (64)	52 ± 1 0

Each value represents the mean of ± S.D. of triplicate determinations

Table 4.2

Immobilization yield of anionic/cationic HRP isoenzymes on cyanogen bromide activated Sepharose*.

Preparation	units bound/g		'η' value
	Theoretical** (A)	Actual*** (B)	(B/A)
Anionic			
Native	225 ± 2.8	150 ± 3.2	0.66
Periodate treated	590 ± 2.4	490 ± 4.0	0.83
Cationic			
Native	450 ±2.5	320 ±2.6	0.71
Periodate treated	1600 ±2.6	1470 ±1.6	0.91

Each value represents the mean ± S.D. of triplicate determinations.

* Each gram Sepharose was treated with 200 mg of cyanogen bromide for 10.0 min. at 4°C.

** Theoretical activity was evaluated by substracting the soluble enzyme activity remaining after immobilization from that added for immobilization.

*** Actual activity of enzyme was determined by assaying an appropriate aliquot of the immobilized enzyme.

Immobilization on Con A support

Significant differences existed in the binding, expression of bound activity and extent of covalent connection form between periodate treated eznzyme and lectin between the cationic and anionic forms. While cationic forms is bound to the Con A matrix in larger amounts, the 'η' value was comparable with that of the anionic form. Periodate treatment lowered the extent of binding however, greater fraction of the periodate treated cationic form was found to be coupled to the support than that of anionic counterpart (Table 4.3). Inview of the very low activity bound and retained the preparations were not subjected to further studies.

Properties of immobilized HRP

Effect of pH-The pH dependence of soluble and immobilized anionic and cationic HRP is shown in Figure 4.4. Both anionic and cationic HRP show a significant broadening of the pH-activity profile on immobilization, indicating a marked increase in the stability towards the pH, the pH optimum (i.e. pH 5.0) however, remained unaltered in both cases. The preparation Sp-NHHRP retained relatively more activity at extremes of pH. As compare to the anionic form the soluble cationic preparation appeared somewhat more stable to alkaline pH. The cationic form also improved its thermal stability to a greater extent than the anionic form on immobilization.

Effect of temperature-The effect of temperature on the reaction rate is shown in Figure 4.5. As evident from Figure that in both anionic and cationic HRP, immobilized preparations exhibited significant broadening of temperature activity profile. Contrary to pH activity profile, the

Table 4.3

Immobilization yield and covalent coupling of anionic/cationic HRP isoenzymes on Con A-Sepharose*.

Preparation	units bound/g		'η' value
	Theoretical (A)	Actual (B)	(B/A)
Anionic			
Native	200 ± 1.9	105 ± 1 2	0 50
Native-manno-pyranoside treated	-	10 ± 1.5	
Native-crosslinked	-	70 ± 1 2	
Periodate Treated	150 ± 2.6	84 ± 2 5	0.56
Periodate-manno-pyranoside treated	-	38 ± 2 0	
Cationic			
Native	350 ±1.6	180 ±2 6	0 51
Native-manno-pyranoside treated	-	19 ± 2.6	
Native-crosslinked	-	135 ±2 0	
Periodate treated	275 ±2.3	160 ±1.6	0 58
Periodate-manno-pyrannoside	-	96 ± 1 6	

Each value represents the mean ± S.D. of triplicate determinations

* Each gram Sepharose contained about 3.0 mg Con A

** Crosslinking was performed using 0 1 % glutaraldehyde for 2 0 h at 4°C
Parenthesis show the precent activity retained after treatment

Figure 4.4

pH activity profile of soluble and immobilized anionic (A) and cationic (B) HRP preparations.

Soluble or immobilized anionic HRP (Approximately 250 u' 's) were incubated in appropriate buffer of indicated pH and assayed as discussed in the text. The buffer used for various pH ranges were sodium acetate (pH 4.0 and 5 0), sodium phosphate (pH 6.0 to 8 0) and Tris HCl (pH 9 0) The final concentration of buffer solutions were 0 01M (o), soluble HRP, (▲), Sp-HRP; (●), Sp-NHHRP.

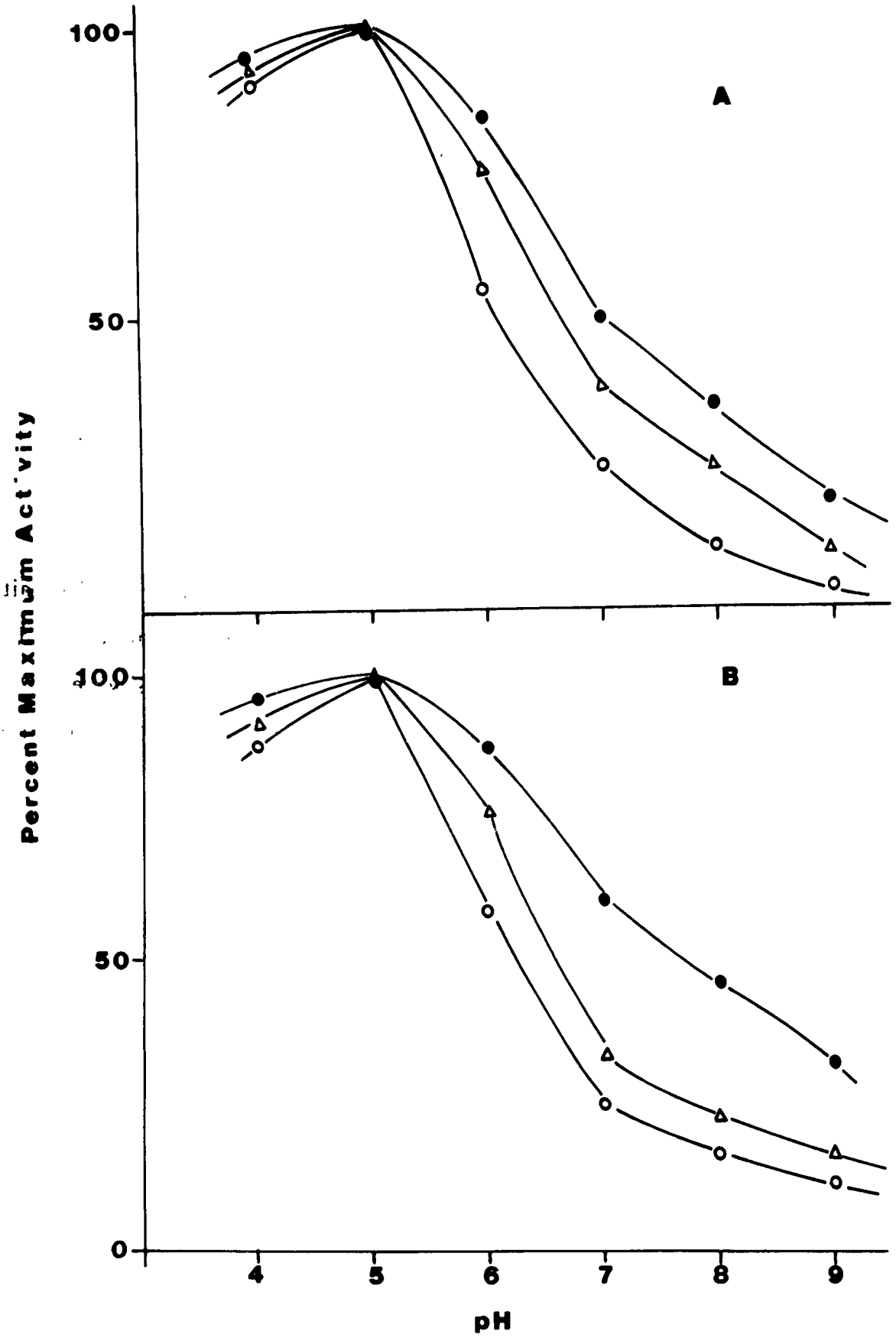
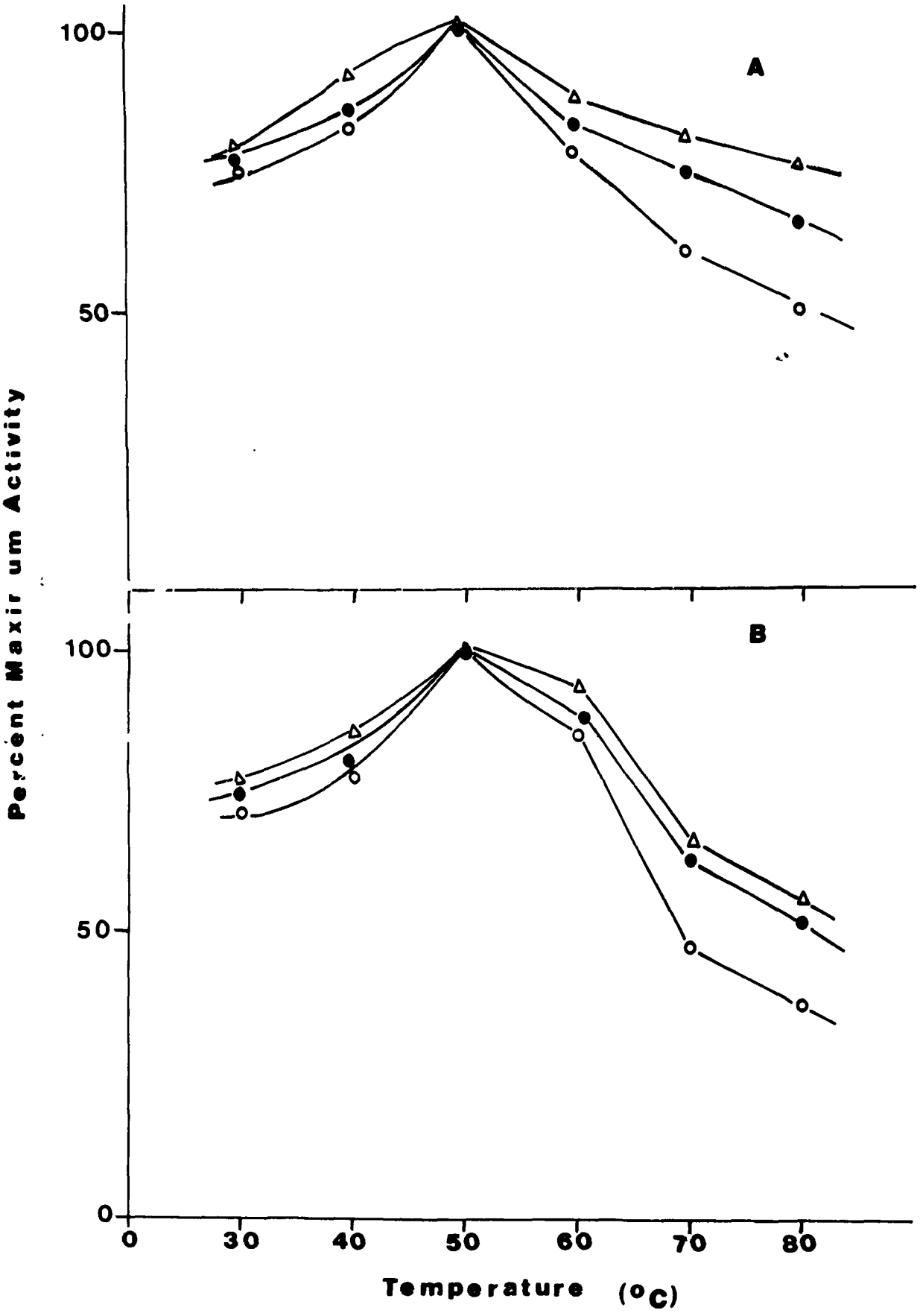

100
50
A
Percent Maximum Activity
100
B
50
0
4
5
6
7
8
9
pH

Figure 4.5

Effect of temperature on the activity of soluble and immobilized cationic (A) and anionic (B) HRP preparations.

Activity of soluble or immobilized anionic and cationic HRP was measeured under standard conditions at the indicated temperature as already discussed in the text (o), soluble HRP, (Δ), Sp-HRP, (•), Sp-NHHRP


100
50
A
100
50
0
B
Percent Maxir um Activity
0
30
40
50
60
70
80
Temperature (°C)

preparation Sp-HRP was found to be the most stable in both cases Although cationic HRP was again found to be more resistant to inactivation at higher temperatures as compared to anionic HRP This suggests that anionic HRP is also more heat labile than cationic HRP (Figure 4.5). The temperature optimum of both forms was found to be 50°C

Thermal stabilities of soluble and immobilized anionic and cationic HRP preparations were further evident from Figure 4 6 Only moderate stabilization was observed by immobilization in case of anionic HRP The soluble HRP lost almost all activity while preparation Sp-HRP and Sp-NHHRP retained about 20 and 14% catalytic activity respectively after incubation for 2 h at 70°C.

On the other hand, cationic HRP shows marked stabilization by immobilization against heat denaturation. As evident from Figure 4.6, at the end of 2 h incubation at 70°C, the soluble cationic HRP retained only 15% enzyme activity whereas preparation Sp-HRP and Sp-NHHRP retained about 40 and 26% activity as a result of similar treatment. These studies clearly indicated that cationic HRP is much more heat resistant than anionic form.

Effect of calcium-Calcium ions have been shown to stabilize many enzymes against heat and other forms of inactivation. As shown in Figure 4.7, when 2mM calcium was included in the preparations during the heat treatment, thermal stability of the native HRP was marginally increased at 70°C, while the immobilized preparations showed remarkable increased in their stability in the presence of calcium in both cases

As shown in Figure 4 7, anionic HRP after 2 h incubation at 70°C, the soluble form retained about only 16% initial enzyme activity while

Figure 4.6

Thermal inactivation of soluble and immobilized anionic (A) and cationic (B) HRP preparations.

Appoximately 100 units of soluble or immobilized anionic and cationic HRP were incbated at 70°C in 0.01M sodium phosphate buffer, pH 7.0. Aliquots were removed at various time intervals, chilled quickly and enzyme activity detemined. (o), soluble HRP; (▵), Sp-HRP, (●), Sp-NHHRP.

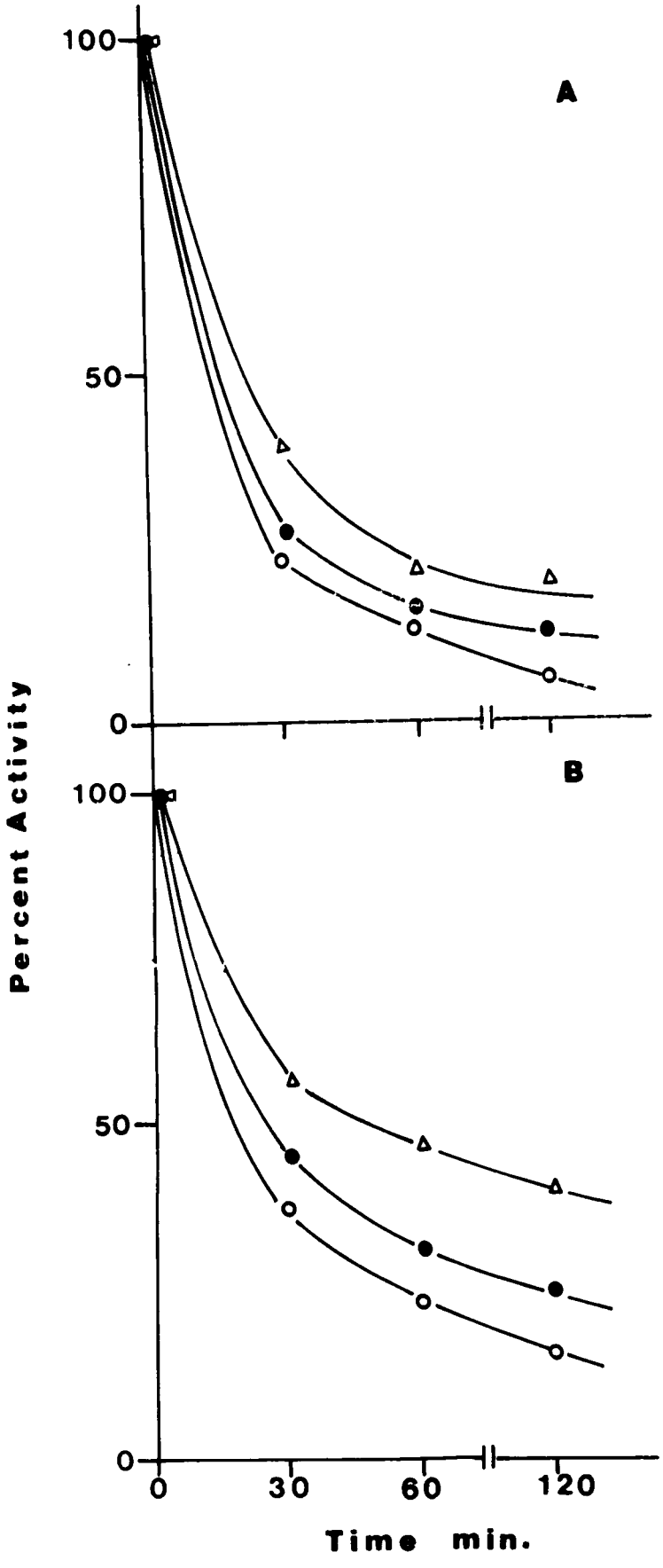
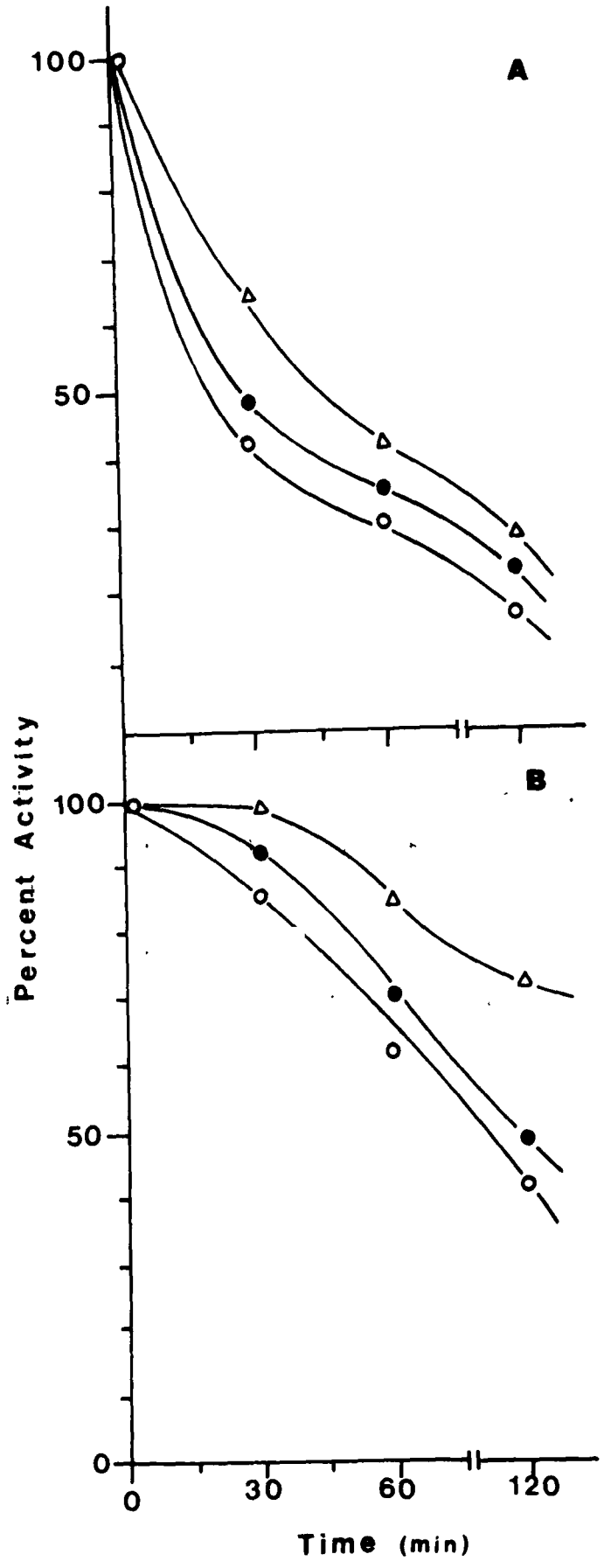

100
50
0
A
B
100
50
0
0
30
60
120
Percent Activity
Time min.

Figure 4.7

Effect of calcium ions on inactivation of soluble and immobilized anionic (A) and cationic (B) HRP preparations.

Approximately 150 units of soluble or immobilized anionic and cationic HRP preparaqions in 0.01M Tris HCl buffer, pH 7 0 were incubated at 70°C for various durations in presence of 2mM calcium ions Aliquots were removed at various time intervals, chilled quickly and enzyme activity determined. (o), soluble HRP; (Δ), Sp-HRP, (•), Sp-NHHRP.


100
50
A
Percent Activity
100
50
0
B
0
30
60
120
Time (min)

preparation Sp-HRP and Sp-NHHRP retained about 32 and 26% initial enzyme activity.

The calcium induced stabilization was also more marked in case of the cationic HRP. Both soluble and immobilized cationic HRP preparations retained far higher fraction of activity at 70°C The soluble HRP retained 40% activity while preparations Sp-HRP and Sp-NHHRP retained 72 and 50% activity respectively when incubated for 2 h at 70°C under identical conditions.

DISCUSSION

In this study, the isoenzymes from crude HRP were isolated using anion and cation exchanger column chromatography. The major isoenzyme forms of horseradish peroxidase are known to contain about 18-20% carbohydrate in the form of oligosaccaharide chains on the surface of the protein molecule (Clarke and Shannon, 1976). The carbohydrate moieties would weaken the interaction between the ezyme and the ion exchanger which results the incomplete separation of isoenzymic forms (Aibara *et al.*, 1982). This necessitated repeated chromatography and after fourth chromatographic separation, the elution profile of each isoenzyme yielded a single protein peaks which were superimposible with peoxidase activity (Figures 4.1 and 4.2).

Theorell and Akeson (1942) reported that each isoenzyme of HRP contained carbohydrate as an integral part of the enzyme Qualitative carbohydrate analysis showed that anionic enzymes were markedly different from cationic isoenzymes. The amino acid composition of these forms was also markedly different and the basicity of cationic form can be attributed to the higher content of the basic amino acid including arginine, lysine and histidine (Shannon *et al.*, 1966).

Sensitivity of the cationic and anionic HRP towards periodate were significantly different. Despite the relatively mild treatment to which the isoenzymes were subjected, both preparations lost significant activity along with glycosyl residues Thus while the anionic form lost about 50% carbohydrate and 38% enzyme activity on treatment with 2mM periodate, its cationic counterpart was depleted by 36% carbohydrate and 24% of the initial enzyme activity. Sensitivity of the peanut anionic/cationic forms of peroxidase to periodate and glycopeptidase F under non denaturing

conditions prompted (Hu and Van Huystee, 1989) to implicate the glycosyl residues in catalytic function. This report, to my knowledge represents only instance in which the oligosaccharides role in catalytic activity has been suggested, although numerous reports on the protective role of glycosyl against denaturation (Goldwasser *et al.*, 1974; Tsuda *et al.*, 1990) and proteolysis (Yamamoto *et al.*, 1987, Olden *et al.*, 1979 ; Merz, 1988) are also available While the data presented here is inadequate to suggest comparable role of glycosyl residues The unusual sensitivity to peroxidation strongly advocates the need for further studies in this regard Indeed increasing functions are being assigned to the glycosyl residues in recent years (Goochee *et al.*, 1992, Rademacher *et al.*, 1988)

Amination with ethylenediamine resulted in greater incorporation of the diamine in cationic form rather than in the anionic form (Table 4.1). Evidently greater fraction of these glycosyl residues are lost in the later and it is not unlikely that larger number of aldehydes are generated in the cationic form. Reaction of periodate with polyols is well known Secondary alcohols yield rise to formic acid while primary alcohols give formaldehyde Single hydroxyl present adjacent to a glycosidic linkage, an O-acetyl, O-sulfate or N-acetylamido group, it is resistant to periodate oxidation (Beeley, 1985). In absence of definite information on the relative structures of the glycosyl of the isoenzymic forms, it is difficult to predict the reasons for the observed difference in sensitivity towards periodate.

As shown in Table 4.3, binding of the HRP isoenzymes with Con A matrix was poor, it could be related due to poor affinity of some isoenzymic form for Con A (Derbyshire, 1973) Lower immobilization than those obtained when HRP was directly coupled to the support was obtained, contrary to observations made earlier on several glycoenzymes those bond to Con A in high yields (Saleemuddin and Husain, 1991) Cationic form of

HRP was immobilized in higher yields than the anionic form yet it was lower than that achieved by direct coupling (Tables 4.2 and 4.3). The low 'η' values obtained and low coupling achieved when the periodate oxidised preparations were added to the Con A matrix, discouraged the use of these preparations for further studies. Immobilization yields of both anionic and cationic HRP on cyanogen bromide activated Sepharose were remarkable increased as a result of periodate and diamine treatment (Table 4.2). This is evidently related as discusssed earlier too, coupling of the HRP enzymes via the amino groups incorporated in the glycosyl residues. The cationic form with higher incorporation of the diamine shows over 3.5 times higher immobilization yield as compared to about 2.5 fold improvement observed in case of the anionic form. It is also interesting to note that the 'η' values of the immobilized periodate-ethylenediamine treated enzymes were markedly higher than, when the native enzyme used for immobilization indicating greater accessibility of these preparations to the substrates. The higher accessibility may be related to their being held relatively away from the support matrix as discussed earlier. It is however, not clear if the preparation is indeed linked both via glycosyl amino groups, amino acid side chain amino groups or only in former.

Soluble cationic form of peroxidase appeared somewhat more stable to alkaline pH and temperature induced inactivation than the anionic form. It is interesting to note stability against alkaline pH is only marginally increased when either of the isoenzyme is immobilized without modification on cyanogen bromide activated Sepharose, while preparations obtained with periodate-diamine adducted were significantly more stable. Among these the cationic form that is presumably linked via greater number of amino groups exhibited clearly greater degree of stabilization while this is agreement with earlier reports that creation of more numerous connections

between enzyme and support resulted in higher stabilization against inactivation (Guisan, *et al.*, 1991; Martinek *et al.*, 1977; Iqbal and Saleemuddin, 1983b). The specific role of interactions of the modified anionic/cationic form with the support matrix can not be ruled out.

Stability conferred by calcium is also remarkable high in case of immobilized cationic form. Haschke and Friedhoff (1978) reported that cationic HRP contains 2.0 moles calcium/mole enzyme, which can be removed by treatment with guanidine hydrochloride and EDTA. Calcium depleted cationic HRP shows decrease in specific activity and thermal stability, indicating the role of calcium in maintaining the protein conformation.

Chapter V

EFFECT OF PH ON THE BINDING AND COUPLING OF GLYCOENZYMES ON *Con* A-SERALOSE

RESULTS

Effect of pH on the periodate induced decrease in glycosyl residues and catalytic activity of some glycoenzymes

With a view to introduce covalent linkages between the glycosyl residues of glycoenzymes and the Con A supports, the glycoenzymes were exposed to periodate oxidation prior to affinity binding on the lectin supports. The role of periodate in oxidative generation of aldehyde groups in glycoenzyme glycosly residues as well as in decreasing the glycosylation is well recognised (Marek *et al.*, 1984). Loss of the glycosyl residues of HRP and invertase was found remarkably dependent upon the pH of the medium during the periodate treatment As shown in Figures 5.1 and 5.2, loss of the periodate induced oxidation of carbohydrate was accompanied by loss of catalytic activity of invertase and HRP. Treatment with 2mM periodate for 30 min. at pH 5.0 resulted in loss of about 20% of the activity of peroxidase while invertase lost only about 12% activity under the identical conditions Biological activity of glucose oxidase and amyloglucosidase was recalcitrant to the periodate treatment and these enzymes suffered no loss of catalytic activity despite the loss of 20 and 30% carbohydrate (Table 5.1).

Effect of pH on the binding and covalent coupling of periodate modified glycoenzymes on Con A-Seralose

In my attempts to introduce covalent linkages between the glycosyl residues of glycoenzyme and Con A-supports, attempts were made to generate aldehyde groups with the help of periodate The amount of enzyme activity bound and that covalently coupled was maximum at pH 5.0 and decreased significantly as the pH was raised to 7 0 both in case

Figure 5.1

pH dependence of the periodate induced effects on the activity and Carbohydrate Contents of HRP

Soluble HRP (Approximately 6000 units) was incubated with 2mM sodium periodate either in 50mM sodium acetate, buffer, pH 5.0 or 50mM sodium phosphate buffers, pH 6.0 and 7 0 for 30 min. in dark at 4°C. The reaction was stopped using 16% ethylene glycol The samples were dialyzed exhaustively against the buffers of appropriate pH before estimation of catalytic activity and carbohydrate content of the enzyme. HRP not exposed to periodate was considered control for calculation of percent activity and carbohydrate.

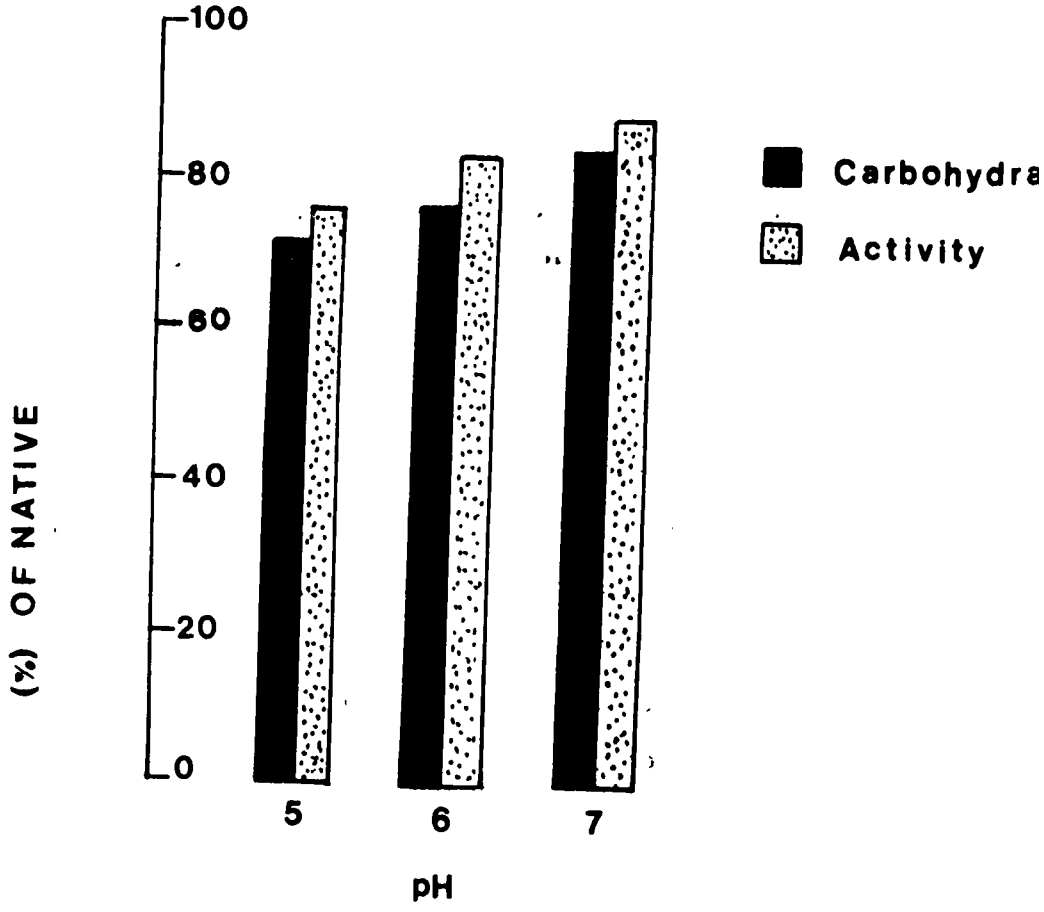

100
80
60
40
20
0
(%) OF NATIVE
5
6
7
pH
Carbohydra
Activity

Figure 5.2

pH dependence of the periodate induced effects on the activity and carbohydrate content of invertase

Soluble invertase (Approximately 4000 units) was incubated with 2mM sodium periodate either in 50mM sodium acetate buffer, 5.0 or 50mM sodium phosphate buffers pH 6.0-7.0 for 30 min. in dark at 4°C. The reaction was stopped using 16% ethylene glycol. The samples were dialyzed exhaustively against the buffers of appropriate pH before estimation of catalytic activity and carbohydrate content of the enzyme. Invertase not exposed to periodate was consider control for calculation of percent activity and carbohydrate.

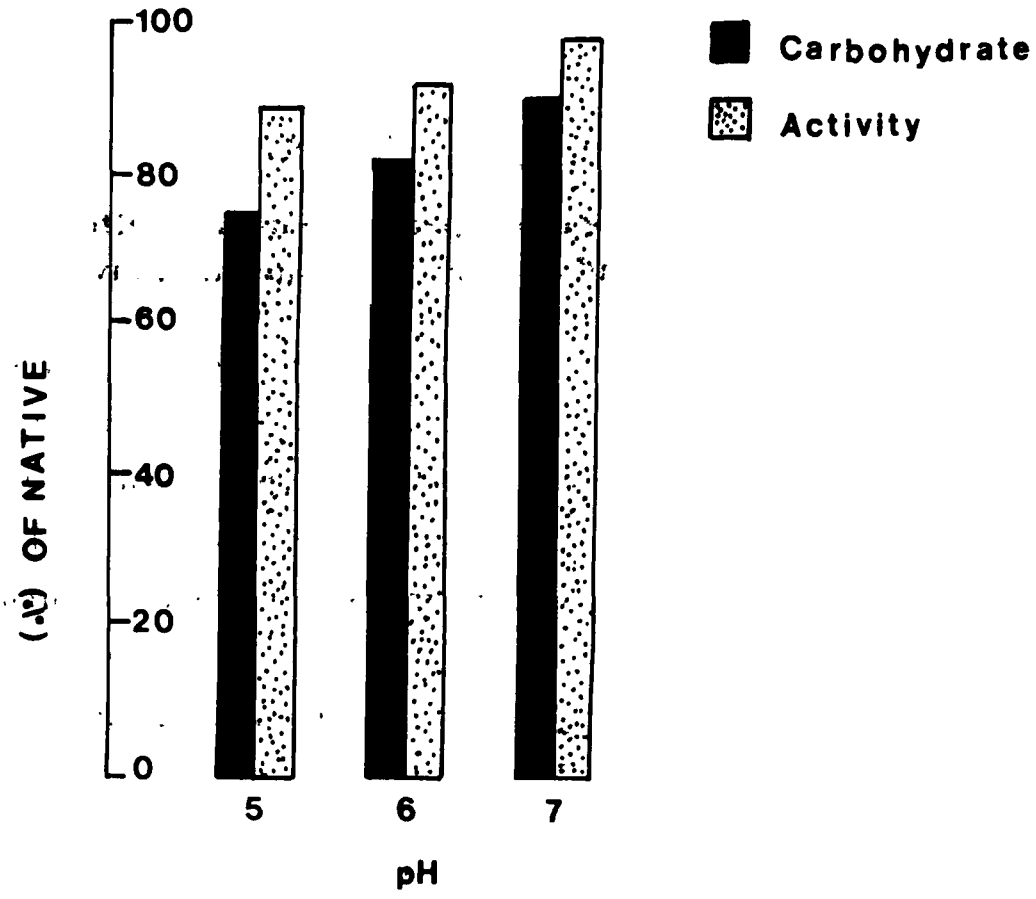

100
80
60
40
20
0
(%) OF NATIVE
5
6
7
pH
Carbohydrate
Activity

Table 5.1

Activity and carbohydrate content of periodate treated glucose oxidase and amyloglucosidase.

Glucose oxidase and amyloglucosidase were treated with 2mM sodium (meta) periodate for 30 min. at 4°C at pH 6.0 in dark. The activity and carbohydrate content were measured after dialysis.

Preparation	Specific activity U/mg protein	Carbohydrate µmoles/mg protein
Glucose oxidase		
Native	210 ± 1.0	1.78 ± 0.06
Periodate treated	198 ± 1.5 (94)	1.65 ± 0.02 (80)
Amyloglucosidase		
Native	690 ± 2.5	1.66 ± 0.02
Periodate treated	670 ± 2.3 (98)	1.14 ± 0.07 (68)

* Each value represents the mean ± S.D. of triplicate determinations.

Value in perenthesis indicate percentage of respective native preparations.

of HRP and invertase (Figure 5.3). This seems to be in agreement with the data presented in Figures 5.1 and 5.2 in which carbohydrate residue loss from glycoenzymes was also maximum at acid pH. It is of interest to point out that HRP and invertase act optimally at pH 5.0. The Table 5.2 shows the immobilization yield of glucose oxidase and amyloglucosidase on Con A - Seralose. Both enzymes show high yield of immobilization on Con A - Seralose even after periodate treatment which is also supported by their high 'η' values. To further investigate if the observed effects of pH were related more to the nature or extent of aldehydic groups generated in the glycosyl residues or to the reaction with immobilized Con A, glycoenzyme exposed to periodate at pH 6.0 were incubated with Con A - Seralose suspended in buffer ranging pH 5.0 to 7.0. Data presented in Figure 5.4, shows that both in case of HRP and invertase the pH of exposure to periodate was less important than that during of binding/coupling to the support. Both enzymes exhibited high immobilization yield and higher covalent coupling at pH 5.0 than at pH 6.0 or pH 7.0.

Effect of glutaraldehyde crosslinking

A control, preparation in which glycoenzymes with unmodified glycosyl residues were bound to Con A - Seralose and crosslinked with glutaraldehyde, was also prepared. For this purpose glycoenzymes bound to Con A - matrix were treated with 0.1% glutaraldehyde for 2 h at 4°C. Such a treatment as shown in Table 5.3, resulted in loss of enzyme activity ranging between 8 to 40%. Maximum and minimum activity being lost by HRP and invertase respectively. Extent of covalent coupling of the glycoenzyme to the support was determined by resistance to

Figure 5.3

The pH dependence of peroxidation, immobilization and covalent coupling of HRP and invertase on Con A-Seralose.

Periodate treated HRP and invertase were mixed with Seralose precoupled with Con A (2.5 mg/gm) in the 0.1M buffers of appropriate pH and incubated for 12 h at 4°C with slow stirring. After immobilization the preparations were centrifuged and washed thoroughly with the buffers. Activity in supernatant, washings and matrix was determined. The extent of covalent coupling of the preparation was determined by treating them with 0.5M methyl α-D mannopyranoside for 12 h at 4°C. Preparations were again centrifuged and washed thoroughly and finally suspended in working buffers

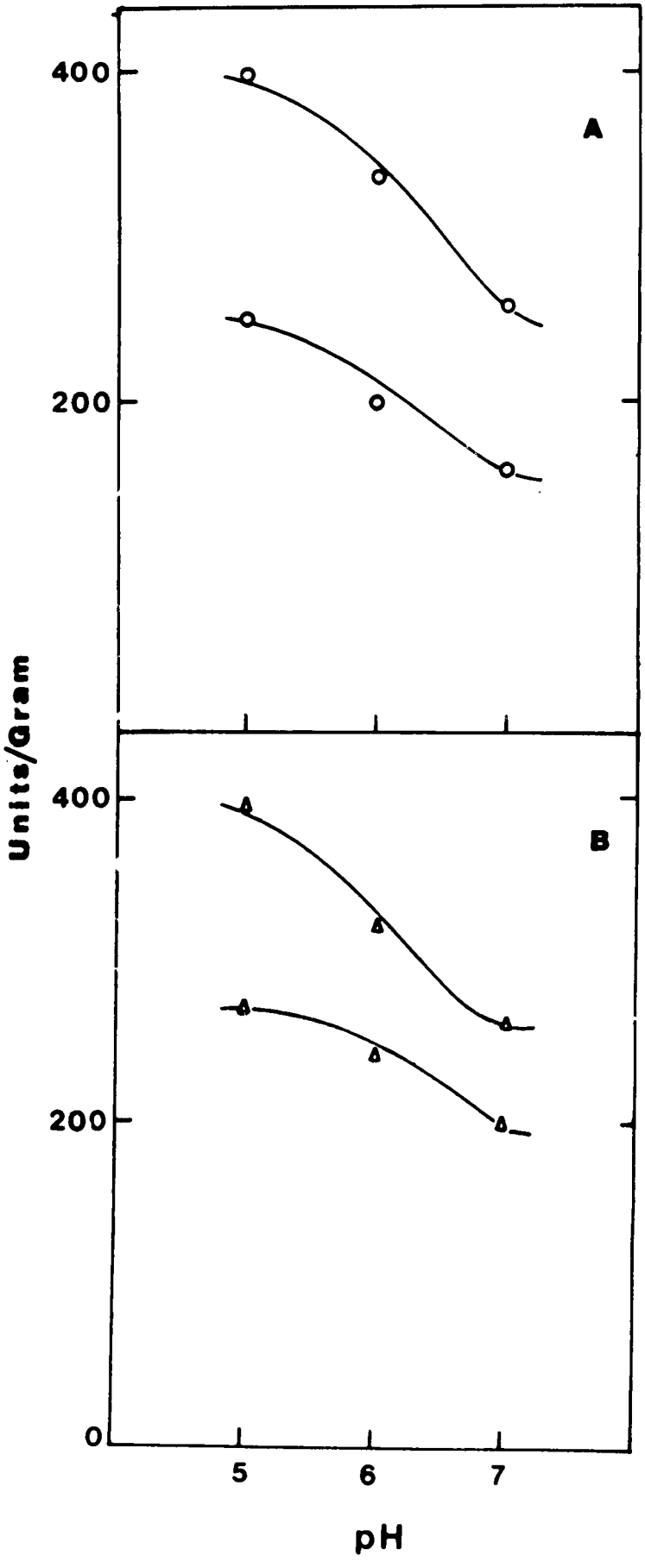

400
200
A
400
200
0
B
Units/Gram
5
6
7
pH

Table 5.2

Immobilization yield of glucose oxidase and amyloglucosidase on Con A - Seralose.

Preparation	units bound/g		'η' value
	Theoretical** (A)	Actual*** (B)	(B/A)
Glucose oxidase			
Native	650 ± 3.0	590 ± 2.8	0.90
Periodate treated*	655 ± 3.5	575 ± 2.0	0.87
Amyloglucosidase			
Native	180 ± 2.8	166 ± 1.8	0.92
Periodate treated*	182 ± 3.0	164 ± 1.5	0.90

Each value represent the mean ± S.D. of triplicate determinations.

* Periodate treatment was given in 0.05M sodium phosphate buffer, pH 6.0 for 30 min. at 4°C.

** Theoritical activity was evaluated by substracting the soluble enzyme activity remain after immobilization from that added for immobilization.

*** Actual activity of enzyme was determined by assaying an appropriate aliquot of the immobilized enzyme.

Figure 5.4

Effects of pH on immobilization yield and covalent coupling of glycoenzymes treated with periodate at pH 6.0 on Con A - Seralose

HRP and invertase were exposed to 2mM sodium (meta) periodate at pH 6.0 and the preparations were incubated with Con A-Seralose in buffers of pH 5.0-7.0. The reaction conditions and procedures for determining the immobilization yield and covalent coupling have been described in the legend to Figure 5.3.

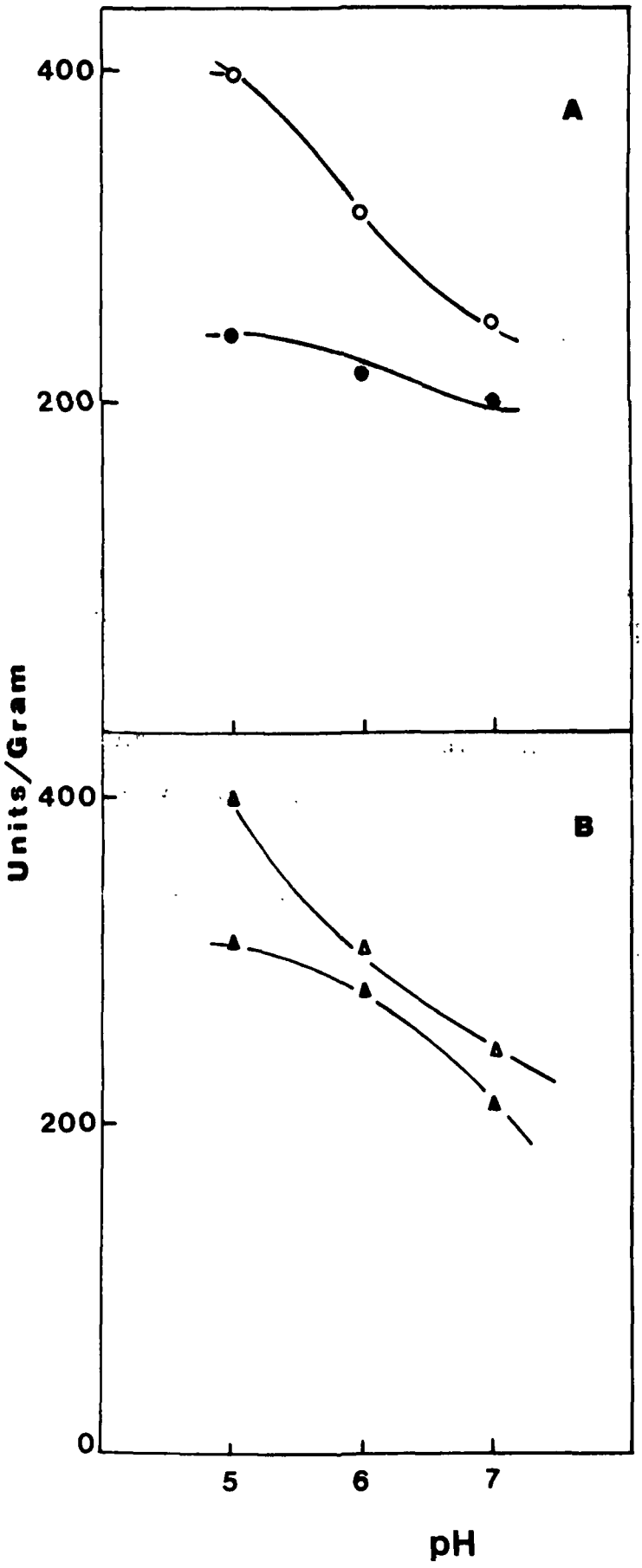

400
200
A
400
200
0
B
Units/Gram
5
6
7
pH

Table 5.3

Effect of glutaraldehyde treatment on the catalytic activity of Con A - Seralose bound glycoenzymes.

Immobilized Enzyme	Activity (A) U/ml	A after crosslinking* (B) U/ml	B after elution with manno--pyranoside (U/ml)
HRP	250	160± 2.0 (60)	138±1.7
Invertase	200	176 ± 1.0 (88)	162±1.8
Glucose oxidase	120	104 ± 2 0 (87)	88±1.8
Amyloglucosidase	166	133 ± 1.1 (80)	177±1.0

Each value represents the mean ± S.D. of triplicate determinations

* The preparations were treated with 0.1% glutaraldehyde for 2 h at 4°C

elution with methyl α-D-mannopyranoside, which also varied in various preparations (Table 5.3).

Thermal stability of immobilized glycoenzymes preparations

Immobilized preparations of HRP, invertase, amyloglucosidase and glucose oxidase irrespective of the nature of linkage between support and enzyme were superior in their resistance to thermal inactivation as compared to the respective native preparations. Among the immobilized preparations those non covalently associated with Con A were generally more labile. This is understandable in view of the possible effects of denaturation on the interaction between the lectin and glycoenzymes. However, the relative stabilities of the preparations linked to Con A support either via carbohydrate or the amino groups differed with different glycoenzymes preparations. Thus while in case of invertase and glucose oxidase the glutaraldehyde linked preparations exhibited higher thermal stability (Figures 5.5 and 5.6), preparations of HRP and amyloglucosidase coupled through carbohydrate chain were more stable (Figures 5.7 and 5.8). Optimum stabilization was observed in case of glucose oxidase preparations covalently linked to Con A - matrix followed by HRP and invertase. Amyloglucosidase exhibited only a small degree of enhancement in resistant to heat inactivation.

Figure 5.5

Effects of heat treatment on soluble and immobilized invertase preparations at 60°C.

Approximately 200 units of soluble and immobilized invertase preparatrion were incubated at 60°C in 0.2M sodium acetate buffer, pH 5.0 for various durations. Aliquots were removed at various intervals, chilled quickly and enzyme activity determined. (o), soluble invertase; (•), Con A bound invertase; (Δ), Covalently coupled invertase to Con A; (▲), Con A bound invertase and crosslinked.

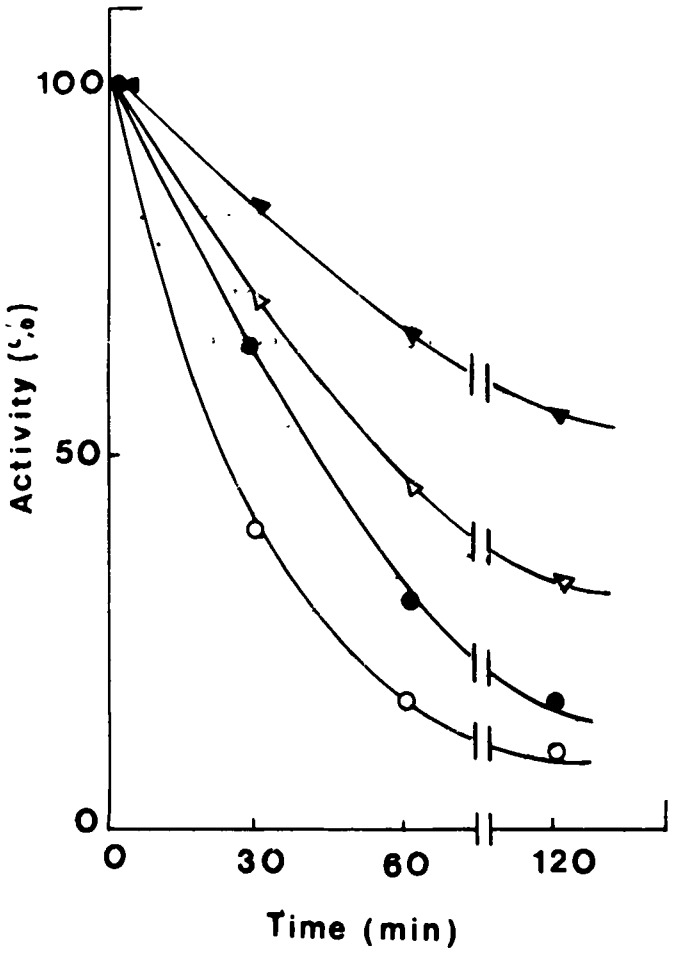

100
50
0
Activity (%)
0
30
60
120
Time (min)

Figure 5.6

Effects of heat treatment on soluble and immobilized glucose oxidase preparations at 60°C.

Approximately 150 units of soluble or immobilized glucose oxidase preparation were incubated at 60°C in 0.1M sodium phosphate buffer, pH 6.0 for indicated durations. Aliquots were removed at various intervals, chilled quickly and enzyme activity determined (o), soluble glucose oxidase; (●), Con A bound glucose oxidase; (▲), covalently coupled glucose oxidase to Con A; (▲), Con A bound glucose oxidase and crosslinked.

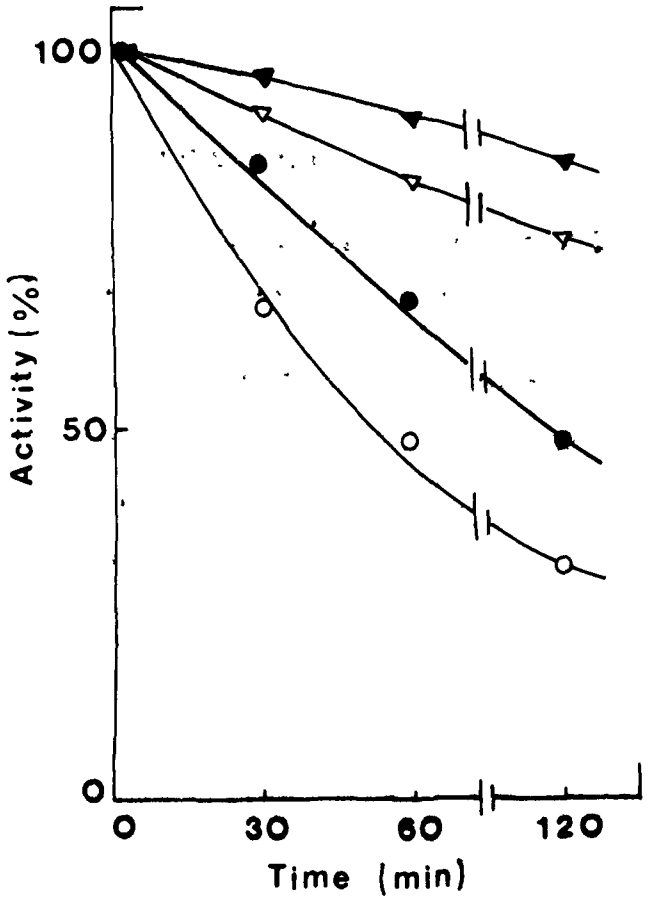

100
50
0
Activity (%)
0
30
60
120
Time (min)

Figure 5.7

Effects of heat treatment on soluble and immobilized HRP at 60°C.

Approximately 200 units of soluble or immobilized HRP preparation were incubated at 60°C in 0.01M sodium acetate buffer, pH 5.0 for indicated durations. Aliquots were removed at various time intervals, chilled quickly and enzyme activity determined. (o), soluble HRP; (●), Con A bound HRP; (Δ), covalently coupled HRP to Con A; (▲) Con A bound HRP and crosslinked.

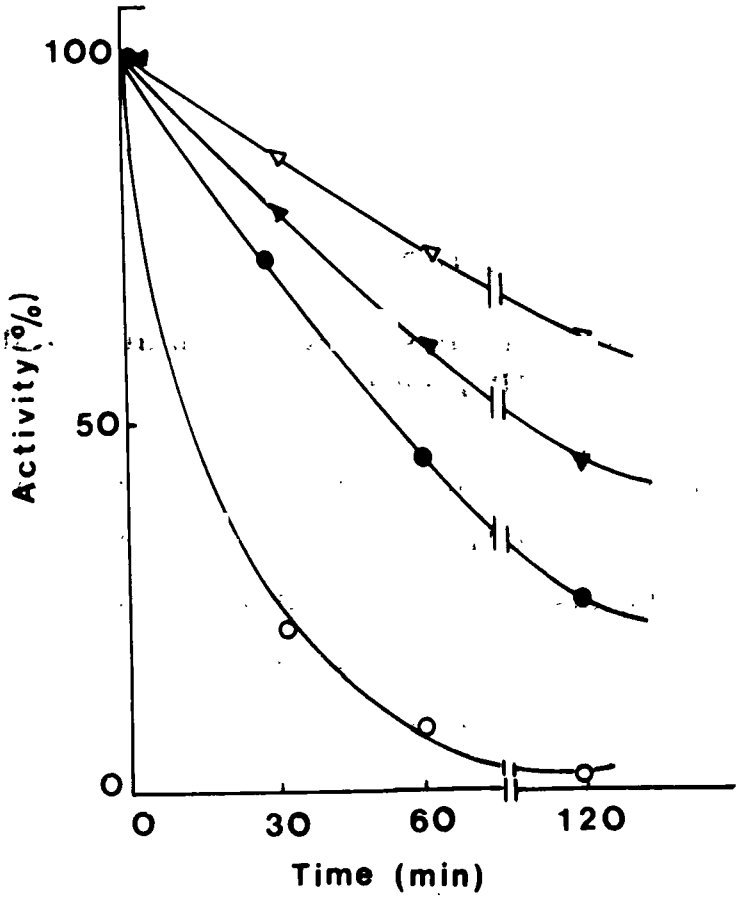
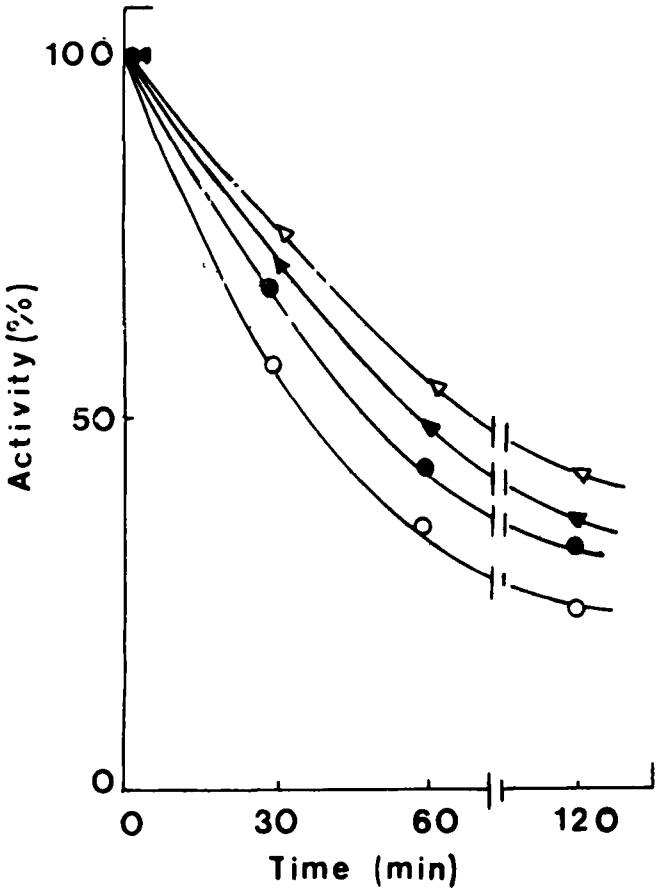

100
50
0
Activity (%)
0
30
60
120
Time (min)

Figure 5.8

Effects of heat treatment on soluble and immobilized preparation of amyloglucosidase at 60°C.

Approximately 100 units of soluble or immobilized amyloglucosidase preparation were incubated at 60°C in 0.1M sodium acetate buffer, pH 5.0 for various durations. Aliquots were removed at various time intervals, chilled quickly and enzyme activity determined. (o), soluble amyloglucosidase; (●), Con A bound amyloglucosidase, (Δ), covalently coupled amyloglucosidase to Con A; (▲), Con A bound amyloglucosidase and crosslinked.


100
50
0
Activity (%)
0
30
60
120
Time (min)

DISCUSSION

Recalcitrance of glycoenzymes to covalent coupling via amino acid side chain amino groups necessitated strategies in which the glycosyl residues form the point of attachment between enzyme and support. Work done earlier in this laboratory (see Saleemuddin and Husain, 1991) and elsewhere has shown the usefulness of Con A supports in improving the immobilization yields of several glycoenzymes, most of which also exhibited superior stability property (Hsiao and Royer, 1979; Woodward and Wiseman, 1978). The principal limitation in the use of Con A matrices, in addition to their high cost, is the inherent risk of desorbtion of the bound glycoenzymes during operation, especially if the substrate/product is a carbohydrate with appreciable high affinity for the lectin. The problem could be overcome to certain extent by introducing covalent linkages between the matrix bound lectin and the bound enzyme (Iqbal and Saleemuddin, 1983a). Crosslinking of the glycoenzymes to the lectin with the bifunctional agent glutaraldehyde resulted in further enhancement in their resistance to various forms of inactivation (Iqbal and Saleemuddin, 1983a; 1983b). This was however, accompanied by appreciably losses in activity of the immobilized enzymes (Iqbal and Saleemuddin, 1983b) presumably resulting from the modification of amino acid side chain amino groups and to certain extent the sulfhydryl groups (Habeeb and Hiramoto, 1968). This is expected to introduce, in addition to intermolecular linkages between lectin and glycoprotein, intramolecular crosslink in the lectin and glycoenzymes. In addition, reagent is expected to modify the reactive side chain amino/sulfhydryl groups without crosslinking where appropriate viscinal groups are not available. However, since glutaraldehyde exists in oligomeric form with variable reach, extensive

inter and intramolecular crosslinking can be anticipated (Richard and Knowles, 1968). This explain the high degree of coupling between glycoenzymes and lectin observed in case of several glycoenzymes preparations. Since glutaraldehyde crosslinking is irreversible, inactivation of the immobilized enzymes amounts to loss of the precious lectin matrix as well. Attempts were therefore, made to introduce covalent linkages between the glycoenzymes and support without introducing intramolecular crosslinks in either. For this purpose aldehydic groups were generated in oligosaccharide chains of the glycoenzyme with the help of periodate prior to affinity binding on the lectin supports.

Periodate treatment resulted in loss of some glycosyl residues of the glycoenzyme which was accompanied by loss of catalytic activity that varied from enzyme to enzyme. HRP was most susceptible to periodate while glucose oxidase and amyloglucosidase were most recalcitrant (Figures 5.1 and 5.2, Table 5.1) while role of glycosyl residues of glycoproteins in the catalytic function of enzyme has not been demonstrated unequivocally, several enzymes do exhibit activity loss in response to periodate exposure (Hu and Van Huystee, 1989). The effects appear to be related to those on amino acid side chain amino groups and are minimized or disappear if the milder periodate treatment is given. Shenoy *et al.*, (1985) have shown in case of amyloglucosidase that removal of carbohydrate with periodate or anhydrous hydrogen fluoride is not accompanied by loss of catalytic activity or antigenecity.

The only report implicating the glycosyl residues in the catalytic function of the enzyme has been made by Hu and Van Huystee (1989). The authors observed marked activity loss in the peanut peroxidase as a result of deglycosylation induced by periodate or with glycopeptidase F.

Periodate induced loss in carbohydrate moieties and catalytic activity

indicated a strong pH dependence with acid pH favouring in both (Figures 5.1 and 5.2). A nearly parallel pH dependence in binding of the glycoenzyme HRP and invertase with modified/unmodified glycosyl was also observed, maximum binding being observed at pH 5.0 and minimum at pH 7.0 (Figures 5.3 and 5.4). Hydrogen ion concentration during the incubation of glycoenzymes with lectin appears crucial as identical pH-dependent binding was observed when the glycoenzymes were treated with periodate at pH 6.0 but incubated with Con A matrix at various pHs. The pH of maximum reactivity of Con A with glycosides polysaccharides and erythrocytes has between pH 6.0 and 7.0, although significant binding takes place between pH 4.0 and 8.0. In case of hydrophobic glycosides like p-nitrophenyl glucosides lectin binding occur optimally between pH 4.0 and 8.0 (Goldstein, 1976) pH values between 6.0 and 7.0 favour the existence of Con A in tetrameric form although dimers are nearly equally effective in glycoside binding (Goldstein, 1976). While the exact nature of linkages between Con A and support has not been ascertained under the conditions employed in this study, the lectin is expected to be associated with several linkages. It is quite likely therefore, that such coupling would prevent the dissociation of Con A into dimers. In order to achieve optimum binding of some glycoenzymes on lectin supports, some workers have found acidic pH values more suitable (Reddy and Shanker, 1989) while with other glycoenzymes pHs around neutrality were more effective (Woodward and Wiseman, 1978). In a recent report on the aggregation of the *Saccharomyces cerevisiae* by Con A, Stratford and Bond (1992) have shown higher degree of aggregation over the pH range of 3.0-5.0. Aggregation described above and below these pH range, the authors believe that the dimeric form is more effective in the yeast cell aggregation than the tetrameric Con A.

Useful preparation of glucose oxidase and amyloglucosidase could be however, prepared at pH 6.0 (Table 5.2). The preparation exhibited high 'η' values suggesting excellent accessibility.

Experiments in which periodate treatment preceeded binding on Con A indicated that the coupling reaction may be slightly more favoured at pH 6.0 and 7.0 as compared to that at pH 5.0. A number of substances with free amino groups react with aldehydic groups in acidic conditions (Roberts, 1978). Reactions of glutaraldehyde was however, favoured at around neutrality although significant reaction also occurs even at pH 4.0 (Habeeb and Hiramoto, 1968). The effect of pH on the native conformation of the glycoenzymes may also be crucial as their incubation with lectin during immobilization last over several hours. Both HRP and invertase act optimally at pH 5.0 and their incubation at pHs far removed from this, pH may disturb their native conformation even couple the enzyme in non native form with the matrix, from which it may not recover even after pH is brought back to the optimum value. The coupling yields of the immobilized enzymes, with the exception of invertase (Figures 5.3 and 5.4) was disappointing. The low coupling may be related to the lack of accessible side chain amino groups for reaction with aldehydic groups of the affinity bound glycoenzymes. Evidently greater number of aldehydic groups are generated in the glycoenzymes with higher glycosylation with greater possibility of reaction with lectin amino groups. Thus for glycoenzymes with small or moderate glycosylation, it may be advantageous to use bifunctional reagents like glutaraldehyde to achieve covalent coupling of most bound glycoenzymes to the lectin.

Stabilization against heat denaturation conferred by binding to Con A support was further improved when the bound enzymes were covalently coupled (Figures 5.5-5.8), this may be related atleast in part to temperature

induced desorbtion of the glycoenzymes from the support. However, these seem to exist no direct correlation between the extent of linkage between enzyme and lectin and improvement in thermal stability. Thus while HRP and amyglocosidase presumably linked via fewer linkages exhibited higher stability than the glutaraldehyde treated preparation (Figures 5.7 and 5.8), with invertase and glucose oxidase it was the later that was more stable (Figures 5.5 and 5.6). Evidently nature of binding and vicinity of the reactive groups in addition to conferring of conformational rigidity are responsible for the stability enhancement effects.

Chapter VI

CHEMICAL MODIFICATION INDUCED EFFECTS ON THE STABILITY OF INVERTASE PRIOR TO AND AFTER IMMOBILIZATION

RESULTS

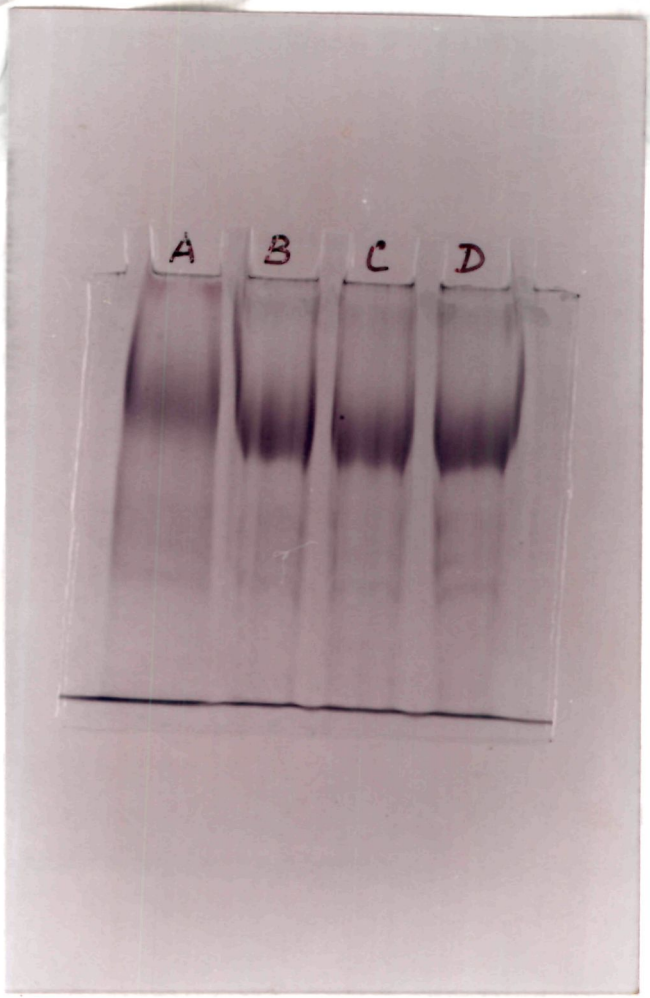
Chemical modification of invertase for immobilization via side chain amino groups and glycosyl residues

In order to generate aldehydic groups in glycosyl residues invertase was treated with sodium (meta) periodate. As shown in Figure 6.1 carbohydrate content of invertase gradually decreased with the length of exposure to periodate which was also accompanied by with a significant loss of carbohydrate residues. About 45% carbohydrate and 30% catalytic activity were lost in 120 min. As the peroxidation may result in formation of intermolecular reaction between invertase molecules due to the formation of Schiffs base between amino groups and aldehydic groups of the enzyme. Invertase subjected to periodate treatment was therefore examined by SDS-PAGE. As evident from Figure 6.2, no aggregation was observed even after 120 min. incubation with periodate. Invertase preparations exposed to longer durations however, migrated with slightly higher mobility than the native enzyme. The following immobilized invertase preparations were obtained (i) Sp-INV, in which native invertase coupled to Sepharose exclusively through amino acid side chain amino groups, (ii) Sp-PEA-INV, in which invertase treated with periodate was reacted with excess ethanolamine to block the generated aldehydic groups. The preparation is also expected to be linked only through amino acid side chain amino groups. The preparation is however, deficient by about 40% in carbohydrate as compared to native invertase, (iii) Sp-PEDA-INV, prepared by treating the periodate treated enzyme with ethylenediamine to block the generated aldehydic groups as well as introduce additional amino groups in the glycosyl residues. Invertase is expected to be coupled both via amino acid side chain amino groups as

Figure 6.1

Effects of periodate on catalytic activity and carbohydrate content of invertase.

Invertase (Approximately 2000 units) was treated with 2mM sodium (meta) periodate in 50mM sodium acetate buffer pH 5.0 for various durations at 4°C in dark. The reaction was stopped with 16% ethylene glycol. Samples were dialysed against the buffer before estimation of activity and carbohydrate. The untreated dialysed sample was considered as control to calculate the percent activity and carbohydrate retained after treatment.


A
B
C
D

well as through amino groups incorporated into carbohydrate moieties, and (iv) Sp-PEDA-TNBS-INV, prepared by treating native invertase with TNBS prior to treatment given by periodate and ethylenediamine. Enzyme is expected to be coupled exclusively through amino groups incorporated into the carbohydrate moieties of invertase as the accessible amino groups have been blocked. Variable losses in activity and carbohydrate were observed during the chemical modification Maximum losses were, 60% activity and 45% carbohydrate, observed in case of Sp-PEDA-TNBS-INV preparation (Table 6.1).

Native, periodate and mono and diamine treated as well as the preparation treated with TNBS prior to periodate and diamine treatment were subjected to PAGE and SDS PAGE containing 5 and 7 5% acrylamide respectively to rule out the possibility of formation of aggregates. As evident from Figures 6.3 and 6.4, no aggregates are formed by this treatment.

Stability properties of the chemically modified invertase

The preparations were also examined for their stability properties prior to immobilization As shown in Figure 6.5, PEDA-TNBS-INV preparation became more labile as compared to native invertase while the preparations PEA-INV and PEDA-INV showed moderate improvement in stability. Maximum stabilization was observed in PEDA-INV preparation.

Immobilization of native and chemically modified invertase

Table 6.2 describes the immobilization yields and 'η' values observed with native and various chemically modified preparaions of invertase. Highest yield of immobilization was obtained in the preparation

Table 6.1

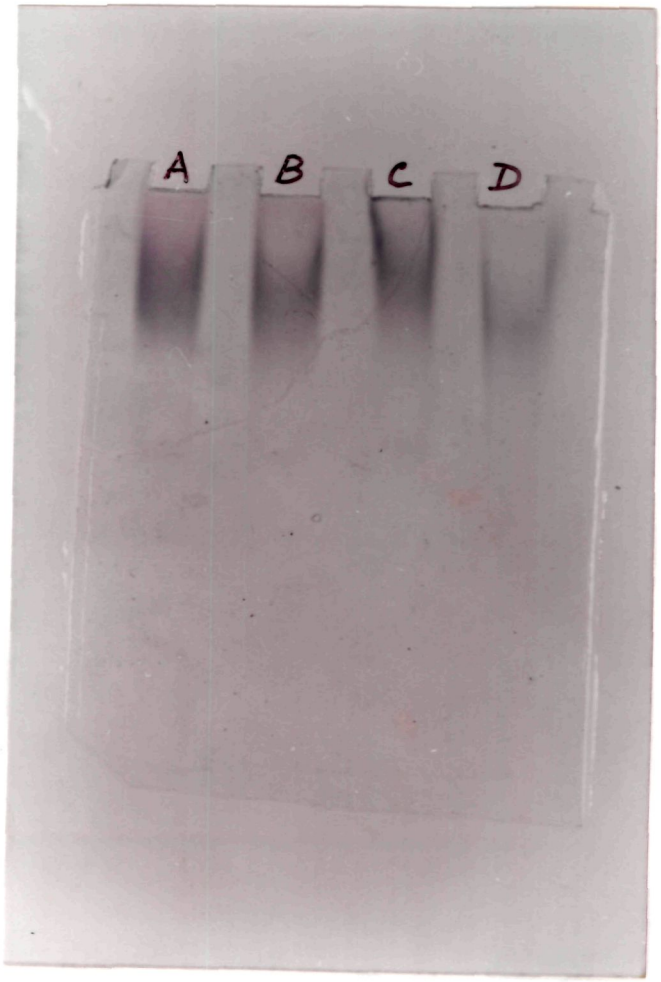
Effect of periodate treatment on activity and carbohydrate content of invertase.

Invertase was treated with 2mM sodium (meta) periodate for 30 min at 4°C in dark which was followed by 100mM ethanolamine or ethylenediamine treatment

Preparations	Specific Activity (U/mg protein)	Carbohydrate μmoles/mg protein	Amino Groups moles/mole protein
Native	5238 ± 2.1	12 8 ± 1 0	12 ± 1.2
Periodate-ethanolamine treated	4000 ± 1.8 (76)	7 8 ± 1 0 (61)	12 ± 1 0
Periodate-ethylenediamine treated	3823 ± 1.0 (73)	8 2 ± 1 1 (64)	23 ± 1 5
TNBS and Periodate-ethylenediamine treated	1316 ± 2.1 (40)	7 ± 1 2 (55)	20 ± 2 0

Each value represents the mean ± S D of triplicate determinations.

Values in parenthesis indicate percentage of respective native preparations


A
B
C
D

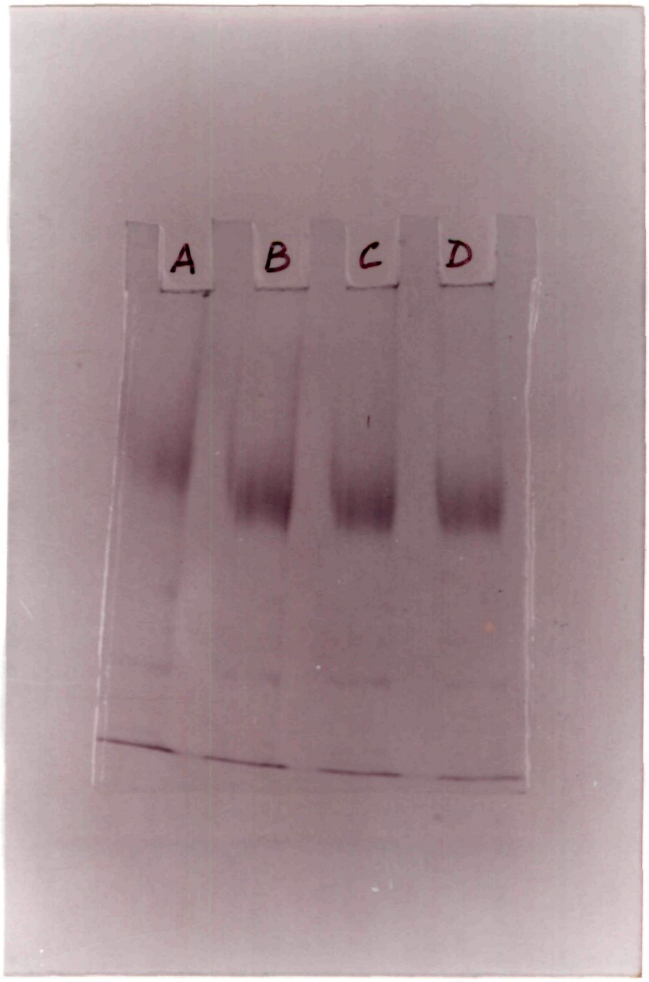

A
B
C
D

Figure 6.5

Thermal inactivation of soluble and various chemically modified invertase preparation at 60°C.

Native and modified invertase preparations (Approximately 100 units) were incubated at 60°C in 0.2M sodium acetate buffer, pH 5.0 for indicated durations. Aliquots were removed, chilled quickly and enzyme activity determined. (o), soluble invertase, (•), periodate-ethanolamine treated; (Δ), periodate-ethylenediamine treated, (*), TNBS and periodate-ethylenediamine treated

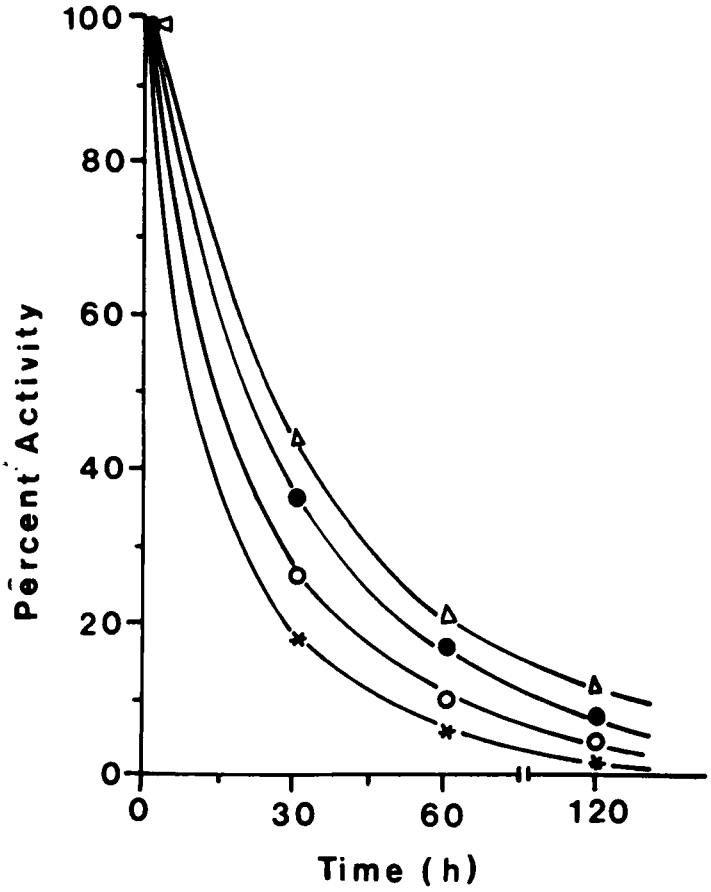

100
80
60
40
20
0
Percent Activity
0
30
60
120
Time (h)

Table 6.2

Immobilization yield of native and modified invertase on cyanogen bromide activated Sepharose.

Preparation	units bound/g		'η' value
	Theoretical (A)	Actual (B)	(B/A)
Native	280 ±1.5	175 ±1 8	0.62
Periodate-ethanolamine treated	525 ±1.7	428 ±2.0	0.81
Periodate-ethylenediamine treated	548 ±1.2	525 ±1.5	0.96
TNBS and Periodate-ethylenediamine treated	285 ±2.5	170 ±1.9	0.59

Each value represents the mean ± S.D. of triplicate determinations.

Theoretical and actual activity was calculated in similar manner as described in table 4.2.

Sp-PEDA-INV that presumably contain invertase bound to support via both of its amino acid side chain and glycosyl amino groups. This preparaion also exhibited highest 'η' value The high immobilization yield and η value of Sp-PEDA-INV were followed by those Sp-PEA-INV, Sp-INV and Sp-PEDA-TNBS-INV preparations, their 'η' values were also obtained in same manner (Table 6.2).

Properties of immobilized invertase

Effect of temperature-The temperature-activity profile of native invertase and various immobilized form is shown in Figure 6.6. Both soluble and immobilized invertase preparaions exhibited a temperature optimum at 60°C except the Sp-PEDA-TNBS-INV preparation which was maximally active at 50°C. The immobilized invertase preparations with the exception of Sp-PEDA-TNBS-INV exhibited a significant broadening of temperature-activity profile. The Sp-PEDA-INV retained maximum activity at 70-80°C.

The thermal stability of these immobilized preparaions is further evident from Figure 6.7, which shows that all immobilized invertase preparations were more stable than soluble invertase

The soluble invertase lost almost all activity in 2 h at 60°C while preparations Sp-INV, Sp-PEA-INV, Sp-PEDA-INV and Sp-PEDA-TNBS-INV preparations retained about 19, 28, 35 and 15% activity respectively under identical conditions. It is interesting to note that the Sp-PEDA-TNBS-INV preparation despite the lability of the TNBS-periodate and diamine treated enzyme, retained higher activity than the soluble enzyme after incubation for various durations at 60°C After incubation for 2 h [illegible]ons Sp-INV, Sp-PEA-INV, Sp-PEDA-INV and Sp-PEDA-TNBS-INV, retained about 18, 30, 35 and 15% activity while their

Figure 6.6

Effect of temperature on soluble and various immobilized invertase preparations.

Approximately 150 units of soluble and various immobilized preparations were incubated as indicated at various temperatures. (o), soluble invertase; (□), Sp-INV; (●), Sp-PEA-INV; (▲), Sp-PEDA-INV; (■), Sp-PEDA-TNBS-INV Please refer to text for details

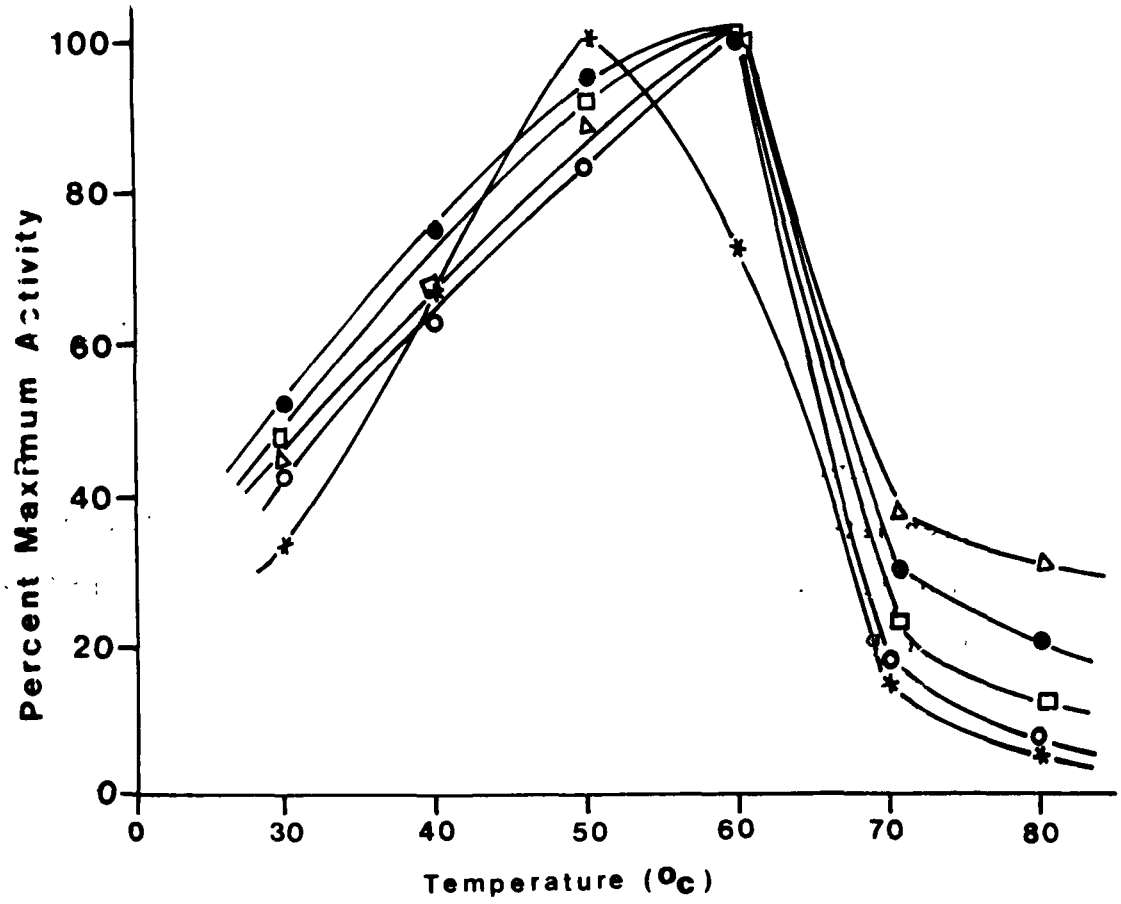
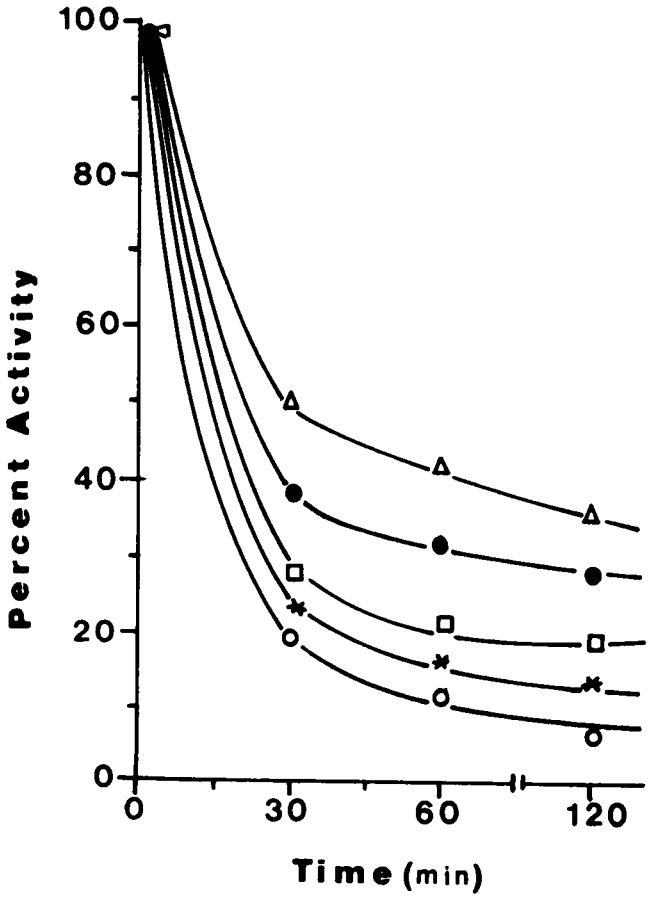

Percent Maximum Activity
100
80
60
40
20
0
0
30
40
50
60
70
80
Temperature (°C)

Figure 6.7

Thermal inactivation of soluble and immobilized invertase at 60°C.

Soluble and immobilized invertase preparations (Approximately 150 units) were incubated at 60°C in 0 2M sodium acetate buffer, pH 5 0 Aliquots were removed at various time intervals, chilled quickly and enxyme activity determined. (o), soluble invertase, (□), Sp-INV; (●), Sp-PEA-INV; (▲), Sp-PEDA-INV; (*), Sp-PEDA-TNBS-INV


100
80
60
40
20
0
Percent Activity
0
30
60
120
Time (min)

soluble forms retained only 5, 8, 10 and 2% activity respectively under similar conditions (Figures 6.5 and 6.6).

Effect of Urea-The effect of urea on native and immobilized invertase was also determined. As shown in Figure 6.8, native invertase retained about 25% activity after incubation with urea for 120 min. while immobilized invertase preparations Sp-INV, Sp-PEA-INV, Sp-PEDA-INV and Sp-PEDA-TNBS-INV preparations retained 55, 45, 49 and 30% enzyme activity respectively. Contrary to heat inactivation studies, the Sp-INV preparation is more recalcitrant to urea inactivation as compared to Sp-PEDA-INV preparation.

The soluble and immobilized invertase preparations were also incubated with varying concentrations of urea (i.e. 2.0M-8.0M) at 37°C for 15 min. At the end of the treatment soluble invertase exposed to 8.0M urea retained only 10% activity while Sp-INV, Sp-PEA-INV, Sp-PEDA-INV and Sp-PEDA-TNBS-INV, retained about 25, 18, 19 and 12% enzyme activity respectively (Figure 6.9).

Figure 6.8

Effect of 3.0M urea on the soluble and immobilized invertase preparations.

Native and immobilized invertase preparations (Approximately 200 units) were incubated in 1 0 ml of 0.2M sodium acetate buffer, pH 4.9 and made 3 0M with respect to urea concentration Aliquots were drawn at the indicated intervals and activity was determined after 10-fold dilution. Untreated sample was consider as control (o), soluble invertase; (□), Sp-INV; (●), Sp-PEA-INV; (△), Sp-PEDA-INV, (*), Sp-PEDA-TNBS-INV

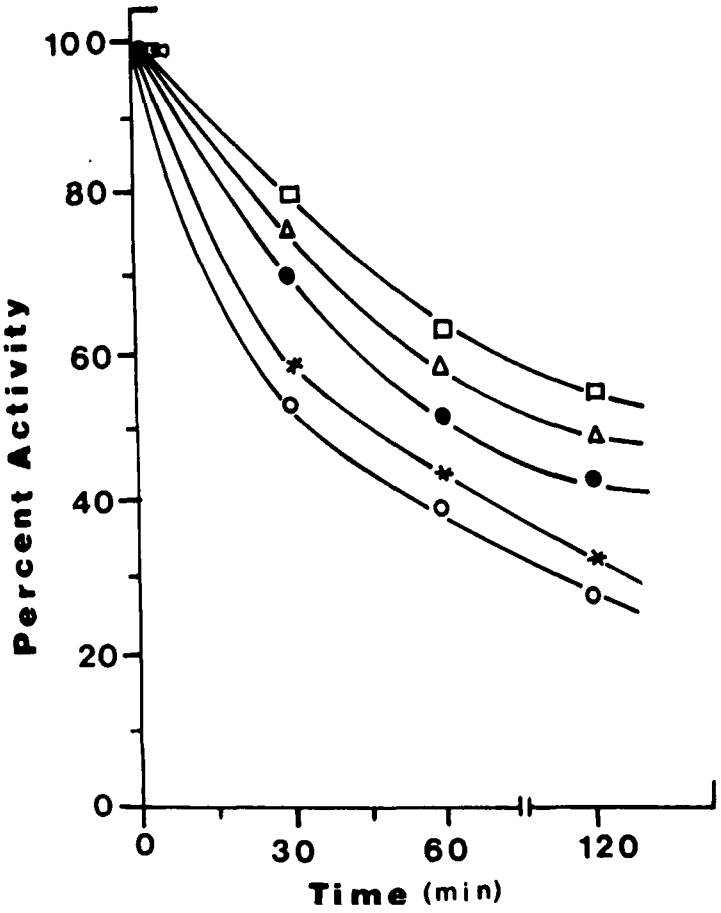
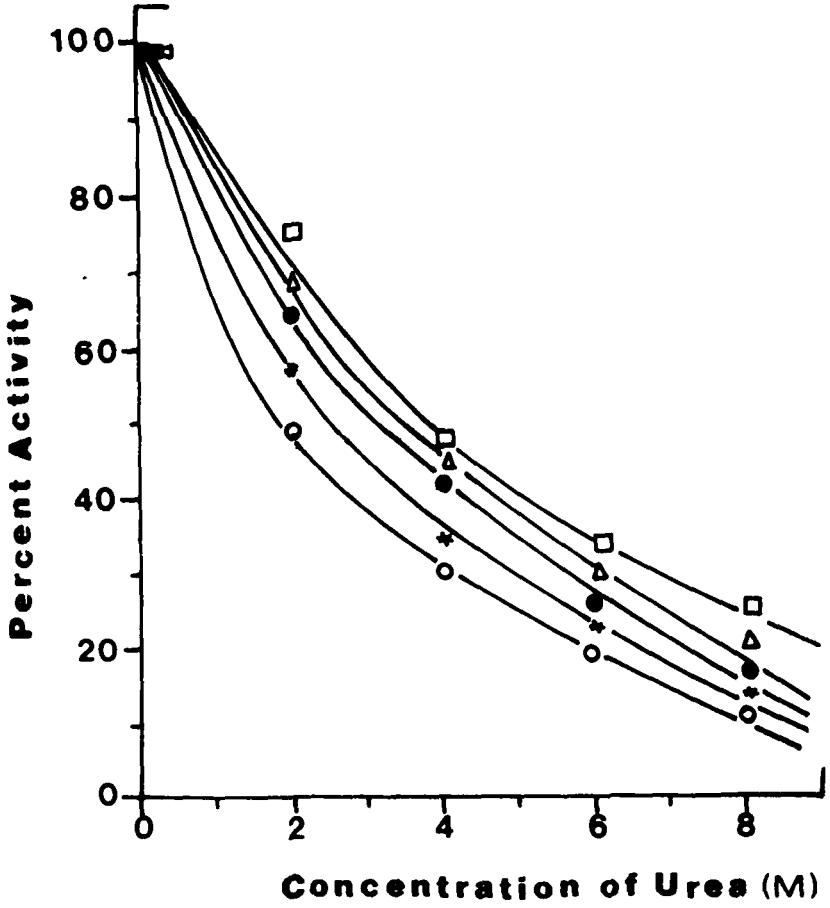

100
80
60
40
20
0
Percent Activity
0
30
60
120
Time (min)

Figure 6.9

Effect of Conc. of urea on activity of soluble and immobilized invertase preparations.

In a total volume of 0 2 ml, native and immobilized prepations of invertase (Approximately 200 units) were incubated for 30 min at 37°C with urea of the indicated concentration At the end of the incubation Period, enzyme activity was determined as already described Activity of the untreated preaparation was taken as 100% (o), soluble INV; (□), Sp-INV, (●), Sp-PEA-INV, (Δ), Sp-PEDA-INV, (*), Sp-PEDA-TNBS-INV


100
80
60
40
20
0
Percent Activity
0
2
4
6
8
Concentration of Urea (M)

DISCUSSION

Yeast invertase with its extensive glycosylation (Trimble and Maley, 1977) provides an excellent model system for glycoenzyme immobilization while extensive information on the immobilization of invertase by a variety of procedures including those via side chain amino groups as well as the glycosyl residues are available (Woodward and Wiseman, 1982; Marek *et al.*, 1984). The relative contributions of the extent and nature of chemical modification to which the enzyme is subjected to prior immobilization have not been examined carefully. Some of these have been addressed in the present study.

Periodate treatment, as also observed earlier, given in order to generate aldehyde groups in the glycosyls of invertase, also caused a time dependent loss in carbohydrate moieties and some losses in catalytic activity (Figure 6.1). No intermolecular crosslinking could be observed during this treatment (Figure 6.2). Early work of Moskowitz and Treffers (1950) and Morgan and Watkins (1951) and several more recent studies (Woodward *et al.*, 1985) have shown that peptide determinants of glycoproteins may be susceptible to very mild periodate treatment. Loss of catalytic activity of yeast invertase exposed to periodate treatment has also been reported by Marek *et al.*, (1984). While earlier studies implicated a role of the glycosyl of invertase in the stability characteristics of the enzymes (Chu *et al.*, 1978).

Since invertase preparations used for immobilization on Sepharose included in addition to the native enzyme, those partially deglycosylated prior to treatment with ethanolamine (PEA-INV) or ethylenediamine (PEDA-INV) or that pretreated with TNBS prior to periodate and ethylenediamine (PEDA-TNBS-INV), their thermal stability and

temperature activity profile were examined. The treatments caused varying degree of invertase inactivation, maximum loss being observed in PEDA-TNBS-INV preparation (Table 6.1) while the TNBS sensivity of the invertase indicate involvement of amino groups catalytic function, retention of 40% activity even after extensive exposure to TNBS suggest operation other mechanisms. Citraconic anhydride and carbodiamide treated yeast invertase also exhibit decreased activity and stability, presumable due to disturbance in the stabillizing salt linkages of the enzyme (Woodward and Wiseman, 1978). Modification of the amino groups with methyl acetimidate that modifies the amino groups without altering the charge caused no significant inactivation or stabilization (Woodward and Wiseman, 1978). In a more recent study Lackband and Langer (1991) have shown that TNBS modification of heparinase can cause 75-100% inactivation, yet amine reaction coupling could be employed for obtaining useful immobilized preparation

Inspite of the potential for chemical aggregation during periodate treatment, resulting from intermolecular Schiffs base formation, no high molecular weight invertase adducts could be observed . Instead a slight increase in mobility of the periodate treated preparation presumably resulting from loss of carbohydrate, could be noted both in native and SDS-Polyacrylamide gel electrophoresis (Figures 6.3 and 6.4). In native gel electrophoresis the TNBS-treated preparation showed slightly faster mobility as anticipated due to increase in the negative charge (Figure 6.3). While all chemical modifications caused invertase inactivation to varying extents, Thermal stability alterations were strongly depended on whether the modification was directed towards the peptide backbone or the glycosyl moieties. Thus while amino group modification by TNBS rendered enzyme labile and toward the temperature optimum, periodate treatment followed

by ethanolamine/ethylenediamine treatment lead to significant increase in thermal stability. While the enhancement in the stability of the partially deglycosylated enzyme is difficult to explains the labilization resulting from TNBS treatment appears to be related to, as discussed earlier to disruption of the stabilizing salt linkages (Woodward and Wiseman, 1978, Suzuki *et al.*, 1966, Usami *et al.*, 1971).

Relative yields of immobilization obtained when various preparations were incubated with cyanogen bromide activated Sepharose Sp-PEDA-INV with its extra amino groups in the truncated glycosyls gave maximum yield presumably due to coupling via these in addition to side chain amino groups. This was followed by Sp-PEA-INV coupled exclusively via side chain amino groups with presumably greater accessibility due to partial removal of the shielding oligosacchride One possible reason for the low yield of the Sp-PEDA-TNBS-INV may be the lack of adequate amino groups for coupling. The 'η' values representing the accessibility, were also quite low in this group presumably due to further disturbances in tertiary structure during immobilization process It is of interest to note that the Sp-PEDA-TNBS-INV preparation has more than 50% of the population in catalytically inactive form, which may also be coupled to the Sepharose matrix along with the active molecules Since enzyme binding has been investigated following only enzyme activity, inactive molecules are not accounted for resulting in low immobilization yields

As evident from Figures 6.6 and 6 7, that all the immobilized invertase preparation are more stable than soluble invertase Maximum stabilization was however, observed in preparation Sp-PEDA-INV followed by Sp-PEA-INV. The Sp-INV and Sp-PEDA-TNBS-INV preparations were least stable As observed earlier with peroxidase the immobilized preparation exhibiting maximum thermal stability was not that resisted

urea denaturation. The Sp-PEDA-INV was much stable against thermal inactivation while Sp-INV resisted urea denaturation better. Since Sp-INV has the glycosyl residues intact (Figures 6.8 and 6.9).

It is tempting to speculate that glycosyl residues of invertase may be important in conferring stability to the enzyme against chemical denaturants.

Chapter VII

SUMMARY

Recalcitrance of glycoenzymes to covalent immobilization procedures necessitated the development of strategies in which carbohydrate residues of the glycoenzymes form the point of attachment between them and support. Usefulness of such procedures in improving the immobilization yields and stability has been demonstrated with several glycoenzymes. Further evaluation of the nature of association of the glycoenzymes with support on the properties of immobilized preparations has been made in this study. For this purpose horseradish peroxidase (HRP), invertase glucose oxidase and amyloglucosidase were variously immobilized and their properties investigated.

Immobilized HRP preparations studied included,(i) Sp-HRP,consisting of native HRP immobilized on cyanogen bromide activated Sepharose (covalently coupled via amino acid side chain amino groups) (ii) Sp-NHHRP,prepared by incubating periodate and diamine-treated HRP with cyanogen bromide activated Sepharose (covalently coupled presumably both via side chain amino groups and those incorporated in glycosyls) (iii) SpNH-COHRP,obtained by reacting the periodate treated HRP with amino Sepharose (covalently coupled via glycosyls) (iv) SpCon A-HRP,obtained by incubating HRP with Con A-Sepharose (affinity bound via glycosyls).

Among the preparations investigated highest immobilization yield and effectiveness factor 'η', indicating high accessibility were obtained with Sp-NHHRP followed by SpCon A-HRP, Sp-HRP and SpNH-COHRP

Sp-NHHRP not only retained higher catalytic activity than Sp-HRP and SpNH-COHRP both at 70°C and 75°C, but also regained greater fraction of enzyme activity when incubated at 0°C subsequent to heat inactivation. Thus while Sp-NHHRP incubated at 70°C for 2 h recovered most of the initial activity, the Sp-HRP and SpNH-HRP regained only 38 and 25 percent.

Immobilized HRP preparations were also superior in their ability to regain enzyme activity after inactivation induced by 8 0M urea Sp-HRP was however,found superior in this respect than Sp-NHHRP, although the later preparation exhibited more marked thermal stability Presence of 10 mM calcium was however, found essential for recovering of enzyme activity after urea inactivation

In order to compare the effects of immobilization on the individual isoenzymic form of HRP, commercial HRP preparation was fractionated and one homogenous anionic and one cationic form isolated Immobilized preparations of both forms, linked via glycosyls as well as amino acid side chain amino groups were prepared. These included, Sp-HRP, Sp-NHHRP and SpCon A-HRP In addition a preparation SpCon A-COHRP in which the affinity bound enzyme covalently linked to the immobilized lectin was also prepared

Periodate treatment given to both isozymic forms to generate aldehydic groups in the glycosyls, resulted in significant loss in activity as well as carbohydrate, although anionic form was found to be more sentitive in this respect

Immobilization conferred additional stability to both isoenzymic forms, although the enhancement in stability was more marked in the cationic form The pH-activity profiles of immobilized HRP exhibited significant broadening suggesting retention of greater fraction of activity at extremes of pH The Sp-NHHRP preparations of both forms were found superior to other immobilized preparations, which inturn retained higher activity than their respective soluble preparations at the pH values far removed from their pH optima

Similarly the improvement in the stability against heat inactivation of the cationic forms of HRP was more marked as compared to those of its anionic counterparts The temperature optima of the preparations however, remained unaltered Thermal stability of Sp-HRP both in case of cationic and anionic forms,Sp-HRP was found to be the highest

Calcium ions were shown to protect the soluble forms of both isozyme forms against inactivation caused by incubation at 70°C, although the effect was more marked in case of the cationic form. Protective effects of the cation were clearly more marked with the immobilized preparations, especially of the cationic form

Yeast invertase contains 50 percent of its weight of carbohydrates and exhibits remarkable affinity for Con A. In addition invertase and some other glycoenzymes bound to Con A supports show marked stabilization against denaturation. Attempts were therefore, made to affinity immobilized invertase, glucose oxidase, amyloglucosidase and HRP on Con A supports and also introduce covalent connections between the lectin and enzyme The later was achieved either by introducing aldehyde groups in the glycosyl chains of the glycoenzymes with periodate prior to immobilization on Con A-Seralose or by crosslinking the immobilized Con A bound glycoenzymes with glutaraldehyde

Periodate induced loss in carbohydrate and enzyme activity exhibited a strong pH dependence with the acid pH favouring both losses Sensitivity of the catalytic activity of various glycoenzymes to the periodate treatment was markedly different; glucose oxidase and amyloglucosidase were less labile as compared to invertase and peroxidase

Acid pH also favoured binding and covalent coupling of the invertase and HRP to the Con A supports, binding and coupling obtained at pH 5 0 were maximum and those at pH 7 0 minimum The pH of medium during immobilization was found crucial than that during periodate oxidation of the glycoenzymes

All immobilized preparations exhibited improvement in thermal stability as compared to the respective soluble preparations. The effect of nature of association between the enzyme and support in conferring additional stability varied among the glycoenzymes investigated Glucose oxidase and invertase bound to the Con A support and linked covalently with glutaraldehyde were more

resistant to heat inactivation than the preparation in which covalent linkages were introduce between the glycosyls and the lectin. On the other hand preparations of invertase and amyloglucosidase linked via glycosyls to the support exhibited higher stability than the glutaraldehyde linked preparations.

Further attempts to generate exclusive connections between the support and either glycosyl residues or amino acid side chain amino groups were also made on invertase. The possible role of chemical modifications and/or carbohydrate depletion to which the enzyme was subjected to was also examined by investigating the properties of soluble modified enzyme. Modifications to which invertase was subjected to included (i) partial depletion of carbohydrate without introduction of any reactive group (achieved by periodate oxidation followed by treatment with the mono amine ethanolamine, PEA-INV),(ii) partial depletion of carbohydrate and introduction of additional amino groups in the side chain (achieved by periodate treatment followed by treatment with ethylenediamine, PEDA-INV), (iii) blocking of the side chain amino acid amino groups followed by introduction of new ones in glycosyls (achieved by the treatment of invertase with trinitrobenzene sulfonic acid,prior to that given to obtain PEDA-INV, TNBS-PEDA-INV).

Periodate treatment caused a loss of approximately 40 percent carbohydrate of invertase and catalytic activity to varing extent depending upon the additional modifications made.No chemical aggregation occured in the periodate-treated invertase as observed with the help of SDS-PAGE.

As compared to native preparations PEA-INV and PEDA-INV exhibited a moderate improvement in thermal stability while a clear labilization was evident in the PEDA-TNBS-INV. When added to cyanogen bromide activated Sepharose the PEAD-INV gave an immobilized preparation that exhibited highest activity and 'η' value.This was followed by preparations containing PEA-INV and PEDA-TNBS-INV.

All immobilized preparations including the Sp-PEDA-TNBS-INV that exhibited lowered activity exhibited, improved thermal stability at 60°C over the native enzyme. The Sp-EDA-INV was found most superior among all those investigated followed by Sp-PEA-INV and Sp-INV. The Sp-INV however, exhibited maximum recalcitrance to urea inactivation among the various immobilized invertase preparations investigated.

Chapter VIII

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Inactivation and reactivation of horseradish peroxidase immobilized by various procedures

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Horseradish peroxidase (HRP) immobilized by coupling the amino acid side chain amino groups or carbohydrate spikes to the matrix has been studied for its resistance to heat, urea-induced inactivation and ability to regain activity after denaturation in order to understand the influence of the nature of immobilization procedure on these processes. The various immobilized preparations were obtained and their properties studied: Sp-HRP was obtained by direct coupling of HRP to cyanogen bromide-activated Sepharose, Sp-NHHRP by coupling periodate oxidized and diamine-treated enzyme to the cyanogen bromide activated Sepharose, SpNH-COHRP by coupling periodate-treated enzyme to amino-Sepharose and SpCon A-HRP by binding of the enzyme on Con A-Sepharose. All the immobilized preparations exhibited higher stability against heat-induced inactivation as compared to the native HRP. Sp-NHHRP was most stable followed by Sp-HRP, SpNH-COHRP and SpCon A-HRP. Sp-NHHRP was also superior in its ability to regain enzyme activity after thermal denaturation, although Sp-HRP regained maximum activity after urea denaturation. Inclusion of Ca^{2+} was essential for the reactivation of all preparations subsequent to denaturation by urea.

Immobilization confers additional stability to a variety of enzymes against several forms of denaturation[1,2] primarily due to their enhanced resistance to unfolding provided by multi-point covalent/non-covalent attachment with the matrix[3,4]. Enzyme immobilization also results in the minimization of protein-protein interactions that account for the irreversibility of enzyme denaturation in response to heat, extremes of *p*H etc. in the absence of chemical modification. Several enzymes that are intrinsically recalcitrant to reactivation subsequent to denaturation in the soluble states refold to the active form under appropriate conditions in the immobilized form[5-7]. It is therefore not unlikely that the observed stability of the enzyme in the immobilized state is related, atleast in part, to their enhanced ability to refold to the native state. While extensive information is available on the effect of immobilization on enzyme stability, studies on the ability of immobilized enzyme to regain activity after inactivation are lacking[6]. This prompted us to study the effect of immobilization of glycoenzyme peroxidase on Sepharose 4B by coupling through carbohydrate spikes and amino acid side chains on its resistance to heat and chemical denaturation and ability to regain activity after such treatment. The results of this study are presented here.

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Materials and Methods

Horseradish peroxidase type I (EC 1.11.1.7), Sepharose 4B and Concanavalin A were purchased from Sigma Chem. Co., USA. *O*-dianisidine-HCl was the product of Centre for Biochemicals, CSIR, India. Hydrogen peroxide, sodium (meta) periodate, calcium chloride and urea were obtained from Sisco, India.

Peroxidation of HRP & preparation of ethylenediamine enzyme adducts—Commercially available HRP (60 mg) dissolved in 20 ml of 0.05 *M* sodium phosphate buffer (*p*H 7.0) was mixed with 5 ml of 50 m*M* sodium (meta) periodate. The mixture was continuously stirred at 4°C for 2 hr in dark. The reaction was terminated by addition of 5.0 ml of 95% ethylene glycol[8]. To the periodate treated HRP was added 0.2 *M* ethylenediamine (*p*H 8.0) to a final concentration of 0.1 *M*. This was followed by the addition of 0.20 ml of 0.1 *M* $NaBH_4$ in 0.1 *N* NaOH for 2.5 hr at 4°C. The unreacted ethylenediamine was removed by extensive dialysis in 0.05 *M* sodium phosphate buffer (*p*H 7.0).

Immobilization of HRP—Various peroxidase preparations were coupled to the cyanogen bromide activated Sepharose using the procedure described by Porath *et al.*[9]. For immobilization of periodate oxidized enzyme, the amino-Sepharose matrix was used. This was prepared by coupling ethylenediamine to the cyanogen bromide activated

Sepharose as described above. Preparation of Con A-Sepharose and binding of peroxidase to the Con A support were performed as described earlier[10].

Peroxidase assay—Peroxidase activities of the native and immobilized preparations were assayed at 37°C by measuring the initial rate of oxidation of *O*-dianisidine-HCl by hydrogen peroxide using the two substrates in saturating concentrations[11]. The immobilized preparations were continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparations. No significant adsorption of the colour complexes formed on the support matrix could be observed.

Other assays—Total carbohydrates were estimated using the procedure described by Dubois[12]. Amino groups of protein were estimated as described by Moore and Stein[13], and protein quantitation by the method of Lowry *et al.*[14].

Results and Discussion

Immobilization of HRP—The commercial HRP was immobilized on Sepharose matrix either via the side chain amino groups or through glycosyl residues and four preparations were obtained: (i) Sp-HRP, a preparation containing HRP coupled to cyanogen bromide activated Sepharose; (ii) Sp-NHHRP, a derivative obtained by coupling periodate treated and aminated enzyme to cyanogen bromide activated Sepharose; (iii) SpNH-COHRP contained periodate oxidised enzyme coupled to amino Sepharose; and (iv) SpCon A-HRP, a preparation containing native HRP bound on Con A-Sepharose. HRP is expected to be bound via amino acid side chain amino groups in Sp-HRP and exclusively. via oxidised glycosyl residues in SpNH-COHRP. The preparation of Sp-NHHRP is likely to be coupled both by amino acid side chain amino groups and those incorporated into the glycosyl residues. Noncovalent affinity binding is expected in the SpCon A-HRP preparation. Periodate treatment under the conditions used for obtaining preparations Sp-NHHRP and SpNH-COHRP, resulted in about 30% loss of catalytic activity and comparable loss in carbohydrates (data not given). Amination of the periodate treated preparation with ethylenediamine resulted in the incorporation of additional 17 moles of amino groups per mole of the enzyme. In view of the high concentration of ethylenediamine used, the possibility of both the amino groups of the diamine reacting with the aldehydic groups of the support is expected to be low. While the involvement of glycosyl residues in the catalytic function of HRP has not been implicated, they seem to be directly involved in the catalytic function of the peanut peroxidase[15]. However, presumably due to its effect on the amino acid side chains, the periodate treatment may cause substantial inactivation of even those glycoenzymes in which the non-involvement of carbohydrates in the catalytic function has been established[16].

Maximum yield of immobilization alongwith highest η value was obtained with Sp-NHHRP. The high immobilization yield may be attributed to the availability of larger number of exposed amino groups for coupling to the support. Since oligosaccharides of HRP are localized on the surface of the enzyme[17], the amino groups incorporated into these moieties are expected to be more accessible than those of the amino acid side chains. Such amino groups, being relatively far removed from the active site of the enzyme, have a greater chance of reacting with cyanogen bromide-activated Sepharose. This contention is supported by the observation that Sp-NHHRP preparation exhibits very high η values, thereby suggesting very high accessibility of the enzyme for the substrate (Table 1). SpCon A-HRP also exhibited high η value as has been observed in case of several glycoenzymes bound to lectin matrix[18]. Contrary to several other glycoenzymes, however, the yield of immobilized peroxidase was very low. This may be related to poor affinity of some HRP forms for Con A[19].

The low immobilization yield and low SpNH-COHRP value may be related to the oligomerisation of the enzyme as a result of reaction of the generated aldehyde groups with the side chain amino groups of other enzyme molecules during the immobilization process. Such reaction may markedly decrease the available aldehydic groups for reaction with the amino groups of the support.

Inactivation and reactivation of HRP—Native HRP is quite stable to heat inactivation. Immobilization, however, conferred additional stability and the immobilized enzyme preparations necessitated rather high temperatures to achieve significant inactivation. As shown in Fig. 1, exposure to 75°C for 30 min completely inactivated native HRP while the various immobilized preparations retained significant enzyme activity. The Sp-NHHRP was most stable and retained 29% activity. Due to the risk of desorption of HRP bound to Con A-Sepharose at high temperatures, the SpCon A-HRP preparation was not used in these studies. The Sp-NHHRP not only retained maximum activity after the heat treatment but also recovered upto 60% of initial activity on incubation at 0°C. Incubation with mixtures of reduced and oxidized glutathione that facilitate recovery of enzyme activity after

Table 1—Immobilization of HRP by various procedures[a]

	Sepharose support	Enzyme	Units bound/g[d]		η value (actual/ theoretical)
			Theoretical	Actual	
(i)	CNBr-activated[b]	Native	1500	900	0.60
(ii)	CNBr-activated	Diamine adducted	2000	1850	0.92
(iii)	CNBr-activated + diamine treated	Periodate treated	1150	600	0.52
(iv)	CNBr-activated + Con A treated[c]	Native	900	800	0.88

a—Each value represents mean of atleast four independent experiments performed in duplicate.
b—Each gram of Sepharose was treated with 200 mg CNBr for 10 min.
c—10 mg Con A was coupled to one gram of Sepharose.
d—About 2500 units of HRP were added to each gram of Con A-Sepharose and units unbound and those in washings were substracted from the added units to obtain "Theoretical" binding An appropriate aliquot of the immobilized preparation was assayed to obtain the "Actual" activity.

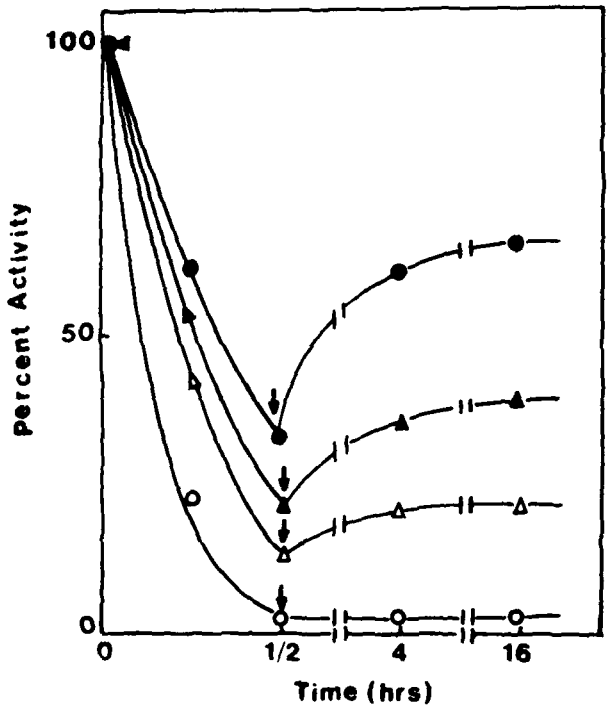

Fig 1—Effect of exposure to 75°C on inactivation and subsequent reactivation of native and immobilized HRP [Approx. 200 units of native or immobilized HRP preparations were incubated in 0 01 *M* sodium phosphate buffer (*p*H 7 0) in a total volume of 1.5 ml for 30 min. The preparations were quickly transferred to ice and further incubated for varying periods. After incubation in ice, the preparations were brought to 37°C and HRP assayed by standard procedure as described in the text. Arrows indicate the time points at which the samples were transferred to ice (○), soluble HRP, (▲), Sp-HRP, (△), SpNH-COHRP, (●), Sp-NHHRP]

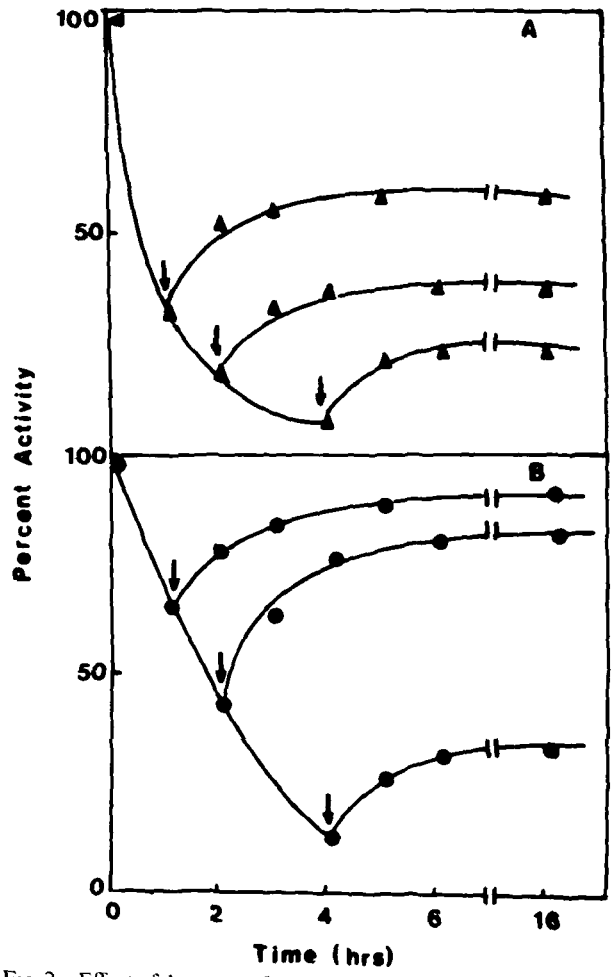

Fig 2—Effect of duration of exposure at 70°C on the inactivation and reactivation of Sp-HRP (A) and Sp-NHHRP (B) [Immobilized preparations Sp-HRP and Sp-NHHRP were incubated at 70°C for varying periods The preparations were subsequently transferred to 0°C for reactivation Arrows indicate the time points at which the samples were transferred to 0°C Various HRP preparations are indicated by symbols as detailed in the legend to Fig 1]

inactivation did not help in increasing the reactivation of heat inactivated HRP (unpublished observations). The ability of the HRP preparations to recover enzyme activity was inversely related to the length of the heat treatment. As shown in Fig. 2, both

Sp-NHHRP and Sp-HRP regained less and less of the initial activity as the length of exposure to 70°C increased. At all the time points investigated, however, Sp-NHHRP retained greater activity after heat treatment and recovered more activity after subsequent incubation at 0°C. A large number of glycoenzymes have been shown to exhibit markedly higher stability if they are immobilized via glycosyl chains rather than amino acid side chains[20-22]. Lack of complete recovery of enzyme activity after denaturation of HRP may be the result of a covalent modification such as hydrolysis of asparagine or entrapment of the enzyme molecules in non-native conformation[6]. It was, however, not possible to investigate the ability of the preparation to recover enzyme activity after milder heat treatment in view of the relatively high thermal stability of the native HRP.

In contrast to the observations made with heat inactivation, Sp-HRP recovered greater fraction of initial enzyme activtiy after urea denaturation (Fig. 3). It was essential to include Ca^{2+} ions in the incubation mixture as very little activity was regained in its absence. Denaturation with guanidine-HCl was shown to dissociate Ca^{2+} from HRP[23]. While it is difficult to explain the superiority of Sp-NHHRP in recovering activity after heat inactivation and of Sp-HRP after urea inactivation, these observations may be related to the difference in nature of linkage with the support. Unfolding achieved by heat treatment and urea denaturation may be qualitatively different as urea treatment is known to cleave all non-covalent linkages, while heat treatment primarily disrupts hydrogen bonds and electrostatic interactions and may actually stabilize hydrophobic forces. Studies of Monsan and Combes[3], and Iqbal and Saleemuddin[22] have shown that stability of the preparation is greatly influenced by the number of linkages between enzyme and support. It is quite likely that Sp-NHHRP may be linked at more sites with the support due to the presence of larger number of accessible amino groups. Such molecules of HRP may not unfold readily and hence resist denaturation.

However, it has been demonstrated by several workers that reactivation of heat denatured enzymes to their native form is facilitated if the polypeptide is first completely unfolded prior to exposing it to conditions that facilitate refolding[24]. The relatively low reactivation yield observed in case of Sp-NHHRP may be related to its inability to unfold completely in urea due to attachment at several sites on the support.

Although conditions prevailing in the enzyme reactors may not be identical to those in the present study, the informations obtained should be useful in attempting to reactivate the enzyme inactivated during continuous operations of the enzyme reactors.

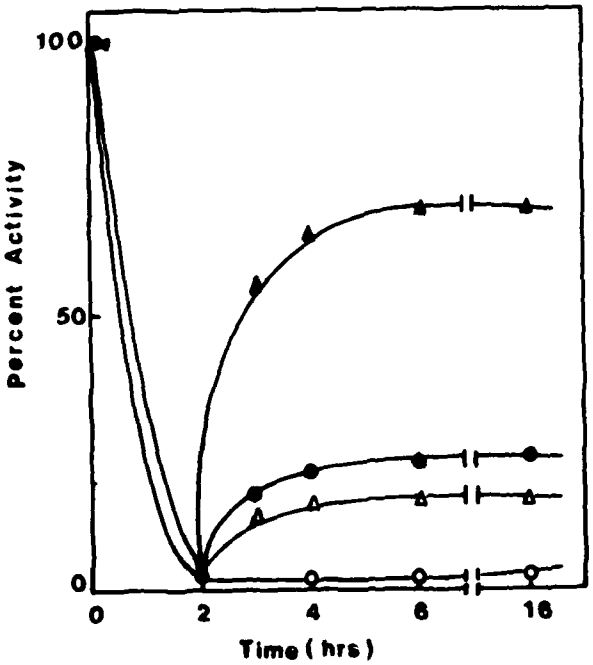

Fig. 3—Reactivation of native and immobilized HRP preparations treated with urea and dithiothretol. [Approx 250 units of native or immobilized HRP preparations in 0 01 *M* Tris HCl buffer (*p*H 7.0) were made 8 0 *M* with respect to urea, 20 0 m*M* with dithiothretol and 5 0 m*M* with EDTA in a total volume of 5 0 ml. The tubes were incubated at 4°C for 2 hr Urea and dithiothretol were rapidly removed from the immobilized preparation by centrifugation and repeated washing with the buffer The preparations were finally placed in 0 01 *M* Tris HCl buffer (*p*H 7 0) containing 2.0 m*M* calcium at 4°C for varying periods as indicated Native HRP was dialysed against 0 01 *M* Tris HCl buffer (*p*H 7 0) containing 2 0 m*M* calcium for 16 hr at 4°C Alternatively the samples were diluted 10-fold to decrease urea concentration and incubated at 4°C for the indicated intervals. Aliquots were removed to determine the activity at varying time intervals (○), soluble HRP, (▲), Sp-HRP, (△), SpNH-COHRP, (●), Sp-NHHRP]

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